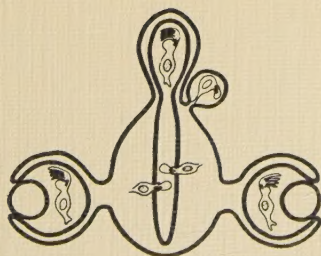


# A STUDY ON THE BASIS FOR ZEITGEBER ENTRAINMENT

With special reference to extra-  
retinal photoreception in the eel

Theo van Veen

Lund 1981





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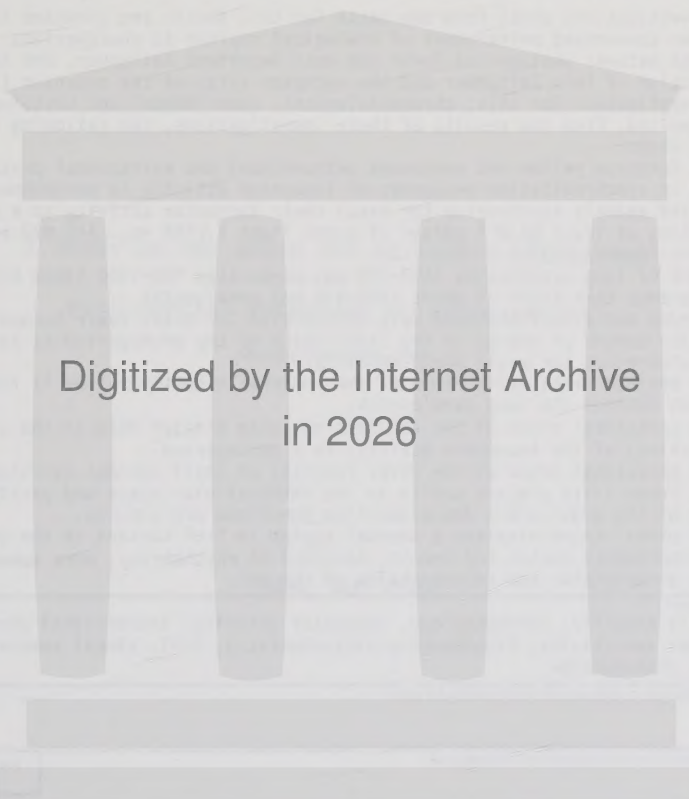
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| Abstract:<br>The investigations which form the basis for this thesis are directed towards the problems concerning entrainment of biological rhythms to photoperiods. It is known that the natural photoperiod forms the most important Zeitgeber, and therefore the interaction of this Zeitgeber and the receptor sites of the organism (photoreceptors) was investigated. For this, chronobiological, experimental and histological methods were applied. From the results of these investigations, the following conclusions can be drawn:<br>1. The European yellow eel possesses extraretinal and extrapineal photoreception involved in synchronization processes of locomotor activity to environmental lighting.<br>2. Intact animals synchronize (or mask) their locomotor activity to a photoperiod containing at least $6 \times 10^{-3} \mu\text{W}/\text{cm}^2$ of green light ( $\lambda 548 \text{ nm}$ , $\Delta\lambda 16 \text{ nm}$ ) and $10^{-2} \mu\text{W}/\text{cm}^2$ of white light ( $2750^\circ\text{K}$ ).<br>3. Light of long wavelengths (650-700 nm) penetrates 100-1000 times better into the hypothalamus than light of short (400-450 nm) wavelengths.<br>4. Blinded and pinealectomized eels synchronize (or mask) their locomotor activity to a similar amount of energy in the light phase of the photoperiod as to which intact eels synchronize (or mask) their activity.<br>5. The maximum sensitivity range of the encephalic photoreceptor is rather broad but does not include the long wavelengths.<br>6. The parapineal organ of the eel does not play a major role in the synchronization (or masking) of the locomotor activity to a photoperiod.<br>7. The parapineal organ of the elver consists of small neurons carrying atypical cilia. These cilia project partly in the intercellular space and partly towards the middle of the organ where desmosome-like junctions are present.<br>8. The pineal organ displays a diurnal rhythm in 5-HT content in the yellow eel.<br>9. Monoaminergic nuclei and tracts, involved in rhythmicity, were demonstrated in the diencephalon and telencephalon of the eel. |                                                                                                                      |
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From the Department of Zoology, University of Lund,  
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A STUDY ON THE BASIS FOR ZEITGEBER ENTRAINMENT

With special reference to extra-retinal  
photoreception



Theo van Veen

LUND 1981

To my family

Karin, Roland, Petra and Sebastian





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This thesis is mainly based on the following papers:

I. Veen, Th. van, Andersson, M., Nilsson, D.-E.:

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testing a computerized system for recording  
biorhythmics. Oikos 1981 (in press).

II. Veen, Th. van, Hartwig, H.-G., Müller, K.: Light-

dependent motor activity and photonegative  
behavior in the eel (Anguilla anguilla L.)  
Evidence for extraretinal and extrapineal  
photoreception. J. comp. Physiol. 111: 209-  
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III. Veen, Th. van, Andersson, M.: Threshold for syn-  
chronization of locomotor activity to visible  
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- IV. Hartwig, H.-G., Veen, Th. van: Spectral Characteristics of visible radiation penetrating into the brain and stimulating extraretinal photoreceptors. J. Comp. Physiol. 130: 277-282 (1979).
- V. Veen, Th. van, Andersson, M.: Extraretinal and extrapineal photoreception in the eel, Anguilla anguilla. Synchronization of locomotor activity to visible radiation of different wavelengths and intensities. MS 1981.
- VI. Veen, Th. van: The pineal and parapineal organ of the elver (Anguilla anguilla L.): A light- and electron microscopic study. MS 1981.
- VII. Veen, Th. van, Laxmyr, L., Borg, B.: Diurnal variation of 5-HT content in the pineal organ of the yellow eel (Anguilla anguilla L.). MS 1981.
- VIII. Fremberg, M., Veen, Th. van, Hartwig, H.-G.: Formaldehyde-induced Fluorescence in the telencephalon and diencephalon of the eel (Anguilla anguilla L.). Cell Tissue Res. 176: 1-22 (1977).

## PREFACE

The investigations which form the basis for this thesis are directed toward the problems concerning entrainment of endogenous rhythms to environmental timing cues (Zeitgebers).

It is known that the natural photoperiod forms the most important Zeitgeber, and therefore the interaction of this Zeitgeber and the receptor site of the organism (photo-receptors) was investigated.

In order to tackle the questions which arose, interdisciplinary methods have been applied.

## INTRODUCTION

### Chronobiology

A salient feature of most, if not all, physiological processes is their cyclic character. Diurnal variations exist in neuroendocrine functions, locomotor activity, feeding, etc. Common for these cyclic parameters is that they often proceed also when time information is abolished. These endogenous oscillators (internal clocks) show a frequency of approximately 24 hours, but yearly and other rhythms are also known (Aschoff, 1963; Bünning, 1973; Menaker et al., 1978). The environmental cue that forces the endogenous oscillator to run with the same frequency as that of the environment is called a "Zeitgeber" (Aschoff et al., 1965). The Zeitgebers, which entrain the endogenous oscillators, usually have an oscillatory frequency of exactly 24 hours, but they show seasonal differences in amplitude and character. The endogenous oscillator is either (1) self-sustained, free-running or (2) fading out if no energy from the environment is restored. Thus, if the Zeitgeber force is abolished, the organism will show its free-running rhythm permanently, or this rhythm fades away if no energy is restored (dampened rhythms; Aschoff and Wever, 1962; Aschoff, 1965). The rhythmic events which organisms display in nature are forced rhythms whose natural (endogenous) oscillation runs parallel to the environmental oscillator.

The interaction of external oscillators, Zeitgebers, and the biological oscillators is dependent on at least two factors: 1) the strength of the Zeitgeber; and 2) the difference between the frequency of the Zeitgeber and the biological oscillator. Even a very strong Zeitgeber can entrain a biological oscillator only within certain limits (range of entrainment; Wever, 1972). A sudden phase shift of the Zeitgeber (changing the light-dark regime, for example, 12 hours) will cause a free-running rhythm until the phase angle of the endogenous rhythm comes within range of entrainment to the Zeitgeber oscillation and a coupling between the two occurs.

Endogenous rhythms can consist of more than one oscillator. The latter are coupled to one another, ranging from firm to loose couplings. Abolition of the Zeitgeber in a multi-oscillator system results in (1) free running if the endogenous oscillators are firmly coupled to one another; (2) splitting of the endogenous rhythm when two or more oscillators or groups of oscillators disintegrate; or (3) apparent arrhythmicity if complete disintegration of loosely coupled oscillators occurs (Aschoff et al., 1975; Eriksson, 1973; Eriksson and van Veen, 1980; Menaker, 1974; Pittendrigh, 1967, 1974).

In order to perceive Zeitgeber information to entrain the endogenous oscillators, the organism needs receptor mechanisms for this Zeitgeber. Daily and seasonal environmental

lighting conditions are regarded as the most important Zeitgebers (Menaker et al., 1978). Therefore, photoreceptor mechanisms and their sensitivity range, adapted to the environmental light milieu, are of great importance to the organism. Except for the dark-to-light and light-to-dark transitions of the daily lighting, other parameters such as temperature, food access, social influences and the like, can act in combination with the daily and seasonal lighting and under some conditions alone as a Zeitgeber. However, these environmental periodicities may exert direct influences on the overt rhythm rather than entraining the underlying oscillation. Phototaxy or social cues can, for example, mask the free-running rhythm (Pittendrigh and Minis, 1964).

#### Extraretinal photoreception in non-mammalian vertebrates

##### The pineal complex

Common for the pineal complex in vertebrates is its site of origin. i.e., the epithalamus primordium of the diencephalon. From more ventrally located parts of the diencephalic neuroectoderm the retinal, and important components of photo-neuro-endocrine systems (cf. Scharrer, 1964) develop.

The epiphysis cerebri of Agnata, Chondrichthyes, Osteichthyes, and Amphibia consist of photoreceptor cells, interstitial



cells of ependymal type (resembling the Müller cells of the retina) and ganglion cells (Meiniel, 1971; Rüdberg, 1968; Oksche, 1971; McNulty, 1978; Falcon, 1979; van Veen et al., 1980). The photoreceptors are functional units for the perception of changes in environmental lighting, and the information gained is transmitted to the brain via the pineal tract (for review, see Dodt, 1973; Ueck, 1979).

Reptiles form a heterogeneous group with regard to pineal structure (Quay, 1979). Some Lacertilians, for example, possess a pineal organ with functional photoreceptor cells, while crocodiles are presently thought to have no pineal structures at all. When a pineal organ is present, the photoreceptive elements can display different stages of regression (Collin, 1969; Oksche, 1970).

The avian pineal organ consists of more or less degenerated photoreceptors, of which no photoreceptive function could be demonstrated (Menaker and Oksche, 1974; Hartwig, 1981).

In most vertebrates investigated, an indoleamine content of the pineal has been demonstrated (for review, see Møller and van Veen, 1981; Reiter, 1978).

The parapineal organ of lampreys and teleosts and the parietal organ present in many lizards and Sphenodon are regarded as homologue structures, whereas the frontal organ in Anura is regarded as part of the pineal organ (Studnička, 1905).

Photoreceptor cells bearing regularly lamellated outer segments are present in the parapineal organ of lampreys (Meinzel, 1971). In different species of teleosts the parapineal organ, which is present in the embryonic stage, is reported to degenerate in adult stage (Holmgren, 1965). Some adult fish, however, exhibit a parapineal organ (Rüdeberg, 1969; Ueck, 1979; van Veen et al., 1980; Wake, 1973; Vigh-Teichmann et al., 1980). The only electron microscopical investigation on the parapineal organ of teleosts (Salmo gairdneri) did show similar cellular elements as those of the pineal organ, but the predominating cell types were neurons and only a few photoreceptor cells were present. The latter exhibited fully developed outer segments.

The parietal eye of lizards is a well-developed photoreceptive organ characterized by an everted retina (for review, see Quay, 1979). The photoreceptor cells are functionally active in light perception (Dodt, 1973).

#### Extraretinal and extrapineal photoreception

As early as 1911, von Frisch showed that the colour change response of European minnows (Phoxinus phoxinus) was not abolished by blinding or pinealectomy. By using light-induced feeding responses on blinded, pinealectomized, and brain lesioned minnows, Scharrer (1928) demonstrated a light sensitivity located in the diencephalon. He proposed

the site for this photoreception to be located in the vicinity of the third ventricle. In this context it is interesting to note that Sacerdote (1971) observed the formation of ectopic retinal structures in the hypothalamic tuberal area of adult crested newts bearing a methylen blue-soaked barrier, indicating the ability of the diencephalon to produce photoreceptive elements.

Encephalic photoreception does not exclusively exist in fish. Benoit (1935; for review, see Benoit, 1964) demonstrated extraretinal photoreception involved in gonadal development in the duck. Using rather coarse quartz rods as light conductors for the photic stimulation of the rhinencephalon, along with suprachiasmatic and paraventricular nuclei, he was able to induce stimulation of gonadal growth in blinded animals. The testes responded exclusively to monochromatic light of longer wavelengths (600-650 nm), Benoit, however, states that the eyes are involved in the process of photoperiodically induced gonadal growth due to the threshold properties of blinded and intact animals. Menaker (1968) gave evidence for extraretinal photoreception in the house sparrow and showed that the eyes do not participate in photoperiodic light perception (McMillan et al., 1975). Yokoyama et al. (1978) showed that testicular growth can be induced by a local illumination (long daylight regime) of the ventromedial hypothalamus by very fine chronically implanted optic fibres, and that short-day illumination of the same area is ineffective in stimulating gonadal growth.

Light-dependent gonadal stimulation and synchronization of locomotor activity by extraretinal and extrapineal photoreception was also demonstrated in the lizard, Anolis carolinensis, by Underwood (1973, 1980). Underwood showed that the eyes are not involved in photoperiodic photoreception. Also, Anura were shown to exhibit extraretinal (see Adler, 1976) and extrapineal photoreception (Cadusseau and Galand, 1980).

Results indicating extraretinal and extrapineal photoreception in Teleostei, Anura and Aves have in part been explained by dermal photoreception (de la Motte, 1964; Adler, 1976). Very strong light flashes seem to evoke electrical responses from isolated skin of every animal investigated, including mammals (Becker and Cone, 1966).

Oksche and Hartwig (1975; see also Hartwig, 1975) using microspectrophotometric techniques, could demonstrate the existence of photolabile compounds in the wall of the third ventricle in Carassius auratus, Salmo gairdneri, Phoxinus phoxinus and tadpoles of Rana temporaria. In this ependymal area of the European minnow, bulbous cilia of the 9x2+0 type were demonstrated by these authors. These bulbous cilia of the sensory type resemble early developmental stages of retinal and pineal photoreceptor outer segments.

Extraretinal photoreception synchronizing locomotor activity rhythms was demonstrated in fish by Eriksson (1972;

Salvelinus fontinalis) and by Siegmund and Wolf (1972; Carassius auratus and Leucaspis delineatus). A tendency of extra-retinal photoreception involved in the control of gonadal activity was shown in Oryzias latipes (Urasaki, 1973).

### Light Milieu of the Aquatic Environment

In fish, the environmental lighting differs greatly from that of land-dwelling animals (Munz and McFarland, 1977; Lythgoe, 1972a), in the following ways: (1) By reflection of the irradiated daylight, the intensity is reduced; (2) The light is polarized; and (3) The light is changed in spectral composition, and its intensity is further reduced depending on the column of water which has to be penetrated and its content of different kinds of particles.

(1) Light reflected from a water surface has approximately the same spectral composition as the light irradiated on the surface. The amount of reflection is greatly dependent on the angle of incidence and on the quality of the water surface (smooth, wrinkles, waves, etc.). Time of the day, season, and geographical situation are factors that strongly influence the aquatic light milieu. These factors accentuate the light regime that the environmental lighting implies, in comparison to the regime of land-dwelling animals, namely the dark-to-light and light-to-dark transients are more abrupt.

(2) Depending on the reflection and incident angle of the daylight, a marked polarization of the underwater light space is present. Depolarization occurs, however, because of scattering of the polarized light by the particles and plankton of the aquatic milieu. Consequently, the ratio of polarized to non-polarized light is greatly influenced by the quality of the water surface, the particle content, and the incident angle of the irradiated daylight.

(3) The modification of the spectral composition of terrestrial daylight to the lighting conditions of the aquatic milieu depends on (a) the absorption characteristics of water cutting off long and short wavelengths, and thus functioning as a monochromator with a broad bandwidth, (b) the content of coloured particles (phytoplankton), and (c) dissolved dyes.

The water composition in respect to content of dissolved and undissolved material is also a factor of great variability. In this respect, ocean water is relatively constant, but seasonal differences occur in the content of phytoplankton. Dissolved salts in ocean water do not influence the spectral composition appreciably. It is of interest that in the deep sea light is also present in the form of bioluminescence. In coastal zones, however, a great variety of factors play a role, i.e., pollution from fresh water systems (industrial or leached substances resulting from rainfall and wind), plus the seasonal variations



of phytoplankton, as mentioned above. Fresh water, however, manifests the greatest variation in respect to spectral composition. An extreme case is Crater Lake (Oregon, western U.S.), which is almost comparable to distilled water. In this type of water, Smith and Tyler (1967) calculated amounts of visible radiation in the range of 400 to 450 nm penetrating with an intensity of  $5 \cdot 10^{-2}$   $\mu\text{W}/\text{cm}^2$  at a depth of 600 m. In extremely contaminated water milieus such intensities can be found in a depth of only a few decimeters.

Regarding the factors that influence the aquatic light milieu, it can be concluded that ocean water gives a more constant and predictable environment, presenting a spectral composition between 430 and 480 nm to relatively great depths (Lockett, 1977). The freshwater milieu is much different, less predictable, and consists of longer wavelengths because of the presence of soluble dyes and pollution. This is also reflected in the retinal pigments of fish. Deep sea fish exhibit maximal sensitivity of their photopigments in the range of 450-490 nm. This differs greatly from pelagic fish and freshwater fish which show maximal absorption in the photopigment systems in the longer wavelengths, i.e., from 490 to 530 nm. Some freshwater fish exhibit photopigments which show maximal sensitivity at even higher spectral wavelengths (up to approx. 690 nm). Probably these fish can perceive deep red light better than the human eye (Lythgoe, 1972b; Bridges, 1972).

Some aspects on the biology of the eel

Although spawning mature eels have never been found in the Sargasso, it is generally accepted that the European eel reproduces in this area of the Atlantic Ocean (at a depth of c. 400 m; Schmidt, 1924). The leaf-shaped larvae of the eel, the smallest of which were caught in the middle of the Sargasso Sea at a depth between 75 and 100 meters, are so different from the adult animals that they originally were described as a separate species, viz., Leptocephalus brevirostris (Kaup). Leptocephali drift with the Gulf Stream towards the coast of Europe, where they arrive at an age of 2 to 3 years (Tesch, 1973). The larvae are then 75-80 mm in length and, except for the eyes, are wholly transparent.

Metamorphosis commences and the leaf-formed body of the leptocephalus larva begins to assume the well-known shape of the eel. The animal is now called elver. The elver, like the leptocephalus larva, is transparent and does not feed during its youngest stage. In Scandinavia (Skagerack and Kattegatt), the elvers arrive between January and April, with a peak in February-March (Lindquist, 1979). The elvers enter the freshwater systems of rivers, channels, and sometimes cross over land through damp grass to ponds. Some remain, however, in the brackish waters of the Baltic Sea and salt water of the North Sea around Denmark.

Pigmentation of the meninx primitiva, skin and blood vessels ensues and the elver starts to feed. Gradually, it assumes the status of a yellow eel.

As in many other poikilothermic vertebrates, the eel is able to change the colour of the skin in response to the background. This colour change is predominantly performed by means of the special cells in the skin containing melanin. When the pigment of these cells is concentrated to a spot, the animal is light in colour and if the melanin is dispersed over the whole area of the melanophores, the eel appears dark. This colour-change mechanism of the eel is under photo-neuro-endocrine regulation, whereas the visual information on background colour is ultimately transduced to hormonal changes influencing the melanophores of the skin. The hormone involved is melanophore-stimulating hormone (MSH) produced in the pars intermedia of the pituitary (for review, see Fremberg, 1978).

The yellow eel will remain in the fresh and brackish waters for a considerable time (5-10 years), but this time lapse could be much longer depending on where the eel lives and on food availability. There is no maturation of the gonads in the yellow eel stage. This occurs only when the eel reaches a new metamorphosis and becomes a silver eel. During this metamorphosis, dramatic changes

occur in the optical system of the animal (Carlisle and Denton, 1959; Gordon et al., 1978). The shape of the head becomes sharper and the eyes become larger. The lens doubles in size and the area of the retina becomes four times the size of that in the yellow eel (hypertrophica). It is in the photopigments of the retina, however, that the greatest changes take place.

The photopigments present in the photoreceptor cells of the retina area, during the yellow eel stage, rhodopsin 501 (max. absorption at 501 nm) and porphyropsin (max. absorption at 523 nm). In the retina of the sea-living silver eel there is no porphyropsin, but instead another photopigment, namely chrysopsin, with a maximum absorption at 482 nm. Rhodopsin 501 is, however, still present in the silver eel. The result of the change of photopigment is an increased sensitivity at a lower wavelength so that the animal is more adapted to deep-sea lighting conditions (Crescitelli, 1972). Chrysopsin is in fact a commonly occurring pigment in deep-sea fish species (Lythgoe, 1972b; Bridges, 1972).

Except for the changes in photopigments, the density of the photopigments of the retina remains the same in comparison to the retina of the yellow eel (Carlisle and Denton, 1959; Gordon et al., 1978). This, together with the enlargement of the lens, greatly increases the possibilities to detect light and discriminate objects at low light intensities.

## SUMMARY AND DISCUSSION (papers I - VIII)

Locomotor activity and Zeitgeber-perception

In fish, locomotor activity recordings have been performed using photocell equipment (Müller and Schreiber, 1967; Eriksson, 1975) for considerable time. The locomotor activity patterns recorded by this method are reliable, but some characteristics of the behaviour pattern of the animal have to be known. This is shown by the locomotor behaviour of a blinded and pinealectomized European minnow (Phoxinus phoxinus) in a tank provided with two photocells, one located below the water surface, the other above the bottom of the aquarium. The animal was kept in an LD 12:12 regime (L 20  $\mu\text{W}/\text{cm}^2$ , white light of 3400°K) with a dusk and dawn period of one hour. The registration of locomotor activity recorded with the photocell placed at a high level of the tank showed a clear diurnal rhythm. The recordings of the photocell placed in a lower position, however, displayed a distinct nocturnal pattern. The total activity registered by both photocells demonstrated an arrhythmic locomotor behaviour (Fig. 1). This fish exhibited a clear diurnal variation in vertical displacement (cf. Eriksson, 1975).

In addition, the ecological demands of the animal have to be fulfilled (within reasonable limits) and the

animal has prior to investigation to be adapted to the experimental conditions, this in order to stabilize entrainment and synchronization in the new environment.

The transmitter of the photocell equipment ought to function at a wavelength that cannot be perceived by the animal, or its intensity must be at approximately the same level as the dark phase of a given light regime. It should be noted that many shallow living fish have a sensitivity of their retinal pigments which makes it likely that deep red light and even far red light is perceived (Lythgoe, 1972b; Bridges, 1972).

A newly designed computerized equipment for the registration and monitoring of locomotor activity (I)\* was designed as flexible as possible in order to be able to alter the investigated variables and keep other variables constant. Special attention was paid to the light source of the equipment in order to be able to alter the intensity, gradually mimicking dawn and dusk with adjustable intervals, general intensity, and spectral composition. The control panel of the equipment is outside of the experimental room, which implicates that the animals investigated get disturbed as little as possible.

\* In the following, roman numerals refer to publications listed under "Contents"



In the first equipment used (II), dusk and dawn periods could not be provided. According to Gwinner (1975), a square (on-off) light regime forms a weaker Zeitgeber than a light regime provided with dusk and dawn periods. This statement is applicable to day active birds. In nocturnal fish, which show shelter behaviour, part of their behaviour is due to negative phototaxis (II; Eriksson and van Veen, 1980). If phase shifts are provided, a square light regime could in fact show the phototactic behaviour by masking the free running rhythm. This might indeed have happened in investigations performed with the first equipment (II), but this does not devaluate the obtained results, since photoreceptive systems have to be involved in order to perceive square light regimes. The dermal photoreception (de la Motte, 1964) reported in the eel could not be confirmed in this study. Instead, evidences were provided that in blinded and pinealectomized animals light perception was mediated by photoreceptive system(s) situated within the skull.

In this study it was also found that the eyes of the intact eels are also involved in the synchronization processes; a light tight cover placed over the skull of the intact eel did not prevent synchronization. However, studies on the threshold values for synchronization of locomotor activity to a photoperiod, showed that the amount of energy in the visible range in the

light phase of the photoperiod necessary for the synchronization of locomotor activity in intact as well as in blinded and pinealectomized eels (III) exceeds the threshold for light detection of retinal photoreceptors of the closely related American eel (Anguilla rostrata) by at least 3 log units (Gordon et al., 1978). The threshold intensities obtained were for white light (2700°K) approximately  $10^{-2} \mu\text{W}/\text{cm}^2$  and for green ( $\lambda$  548 nm,  $\Delta\lambda = 16$  nm)  $6 \times 10^{-3} \mu\text{W}/\text{cm}^2$ . Approximately the same intensities of blue violet ( $\lambda$  403 nm,  $\Delta\lambda = 16$  nm) and red 665-700 nm light failed to synchronize locomotor activity.

Spectral transmission recordings of visible radiation penetrating into the brain (IV) of the eel and several other vertebrates were performed in order to analyse the amount and spectral composition of the penetrating radiation. These recordings performed on supravital material showed that the quality of the light penetrating into the brain is approximately the same in the different species investigated. However, the absolute amount of light penetrating into the brain is depending on the actual size of the skull and the brain.

Photons of long wavelengths (700-750 nm) penetrate approximately 1,000 times more effectively than short wavelengths (400-450 nm). The melanophore index of the eel only effects the absolute amount of light penetrating into the hypothalamus, not the spectral composition.

By exposing blinded and pinealectomized eels to light intensities in the light phase of the photoperiod (LD 12:12) slightly above intensities of radiation at  $\lambda 548 \text{ nm}$  ( $\Delta \lambda = 16 \text{ nm}$ ) to which 50% of the intact eels synchronized their locomotor activity, 4 out of 6 synchronized their locomotor activity to the photoperiod (V). This shows that the threshold for synchronization of blinded and pinealectomized eels is in the same order of magnitude. By correcting the energy content of the different applied wavelengths to contain an equal number of photons reaching the hypothalamus, it was demonstrated that the spectral sensitivity of the extraretinal and extrapineal photoreceptor is rather broad. Extraretinal photoreception has also been demonstrated in the lake chub (Kavaliers, 1980).

According to these results dealing with threshold sensitivity of the extraretinal and extrapineal photoreceptor one might question the involvement of the eyes in Zeitgeber entrainment by reanalysing the results reported in the first investigation (II), where involvement of the eyes was reported.

The coverings on intact animals naturally could not include the orbital cavities and lateral parts of the skull, as in the blinded animals. In the latter, the orbital cavities were filled with aluminium-foil balls and covered with aluminium-foil as well as the skull, including the lateral parts.

Considering the high sensitivity of the encephalic photoreceptor, enough light could have penetrated from aside the aluminium-foil cover as well as through the orbital cavities and lateral wall of the head. Reflected light from the bottom of the recording tank was similar for both the blinded and intact group.

It is therefore possible that the eyes are not involved in the synchronization processes of the locomotor activity to the photoperiod.

Similar reports have been made regarding the photoperiodic gonadal response in the house sparrow (cf. Menaker and Underwood, 1976) and in the lizard Anolis (Underwood, 1980). In these species, however, the eyes seem to be involved in the synchronization processes. In fish an extraretinal photoperiodic gonadal response is reported in the medaka (Urasaki, 1973) and a very strong response is demonstrated in the three-spined stickleback (Borg, 1981). An extraretinal and extrapineal mediated photoperiodic gonadal growth has not been demonstrated previously in teleosts.

#### Photoneuroendocrine systems of the diencephalon

The photoneuroendocrine systems (Scharrer, 1964) include in the eel the pineal organ, parapineal organ, paraventricular organ and other monoaminergic structures of the diencephalon, the encephalic photoreceptor and retinal

projections (retinohypothalamic projection). These systems are involved in integration of extrinsic and intrinsic information necessary for the control of autonomic functions.

That the pineal organ of the European eel (VI) contains photoreceptors was first demonstrated by Oksche and Vaupel-von Harnack (1965). In a later ultrastructural study by R  deberg (1971), the pineal organ was further investigated and the parapineal organ was demonstrated light microscopically. This part of the pineal complex has been regarded to exist only in young teleosts and only sporadically in adults. During the last years the parapineal organ has been demonstrated in several adult fishes (R  deberg, 1969; Ueck and Kobayashi, 1979; van Veen et al., 1980; Vigh-Teichmann et al., 1980). Recently, this organ was found also in adult European minnow (van Veen and Borg, unpublished results) (Fig. 3).

Because of the reported content of few photoreceptors with fully developed outer segments in the parapineal organ in Salmo (R  deberg, 1969), its possible role in extraretinal and extrapineal photoreception has to be considered.

By studying the description of the lesion experiments performed on the European minnow by Scharrer (1928), it can be noted that the reported extraretinal and extrapineal

photoreception also excludes the parapineal organ as being site of the extraretinal photoreceptor. Also in the eel, the parapineal organ does not seem to play a major role in Zeitgeber perception (V).

In the glass eel (elver), photoreceptors with fully developed outer segments are not present in this organ (VI). However, small neurons carrying atypical cilia of sensory type are present. No conclusion about function, other than sensory, can be drawn from the morphology. An investigation of the function of this organ in teleosts is therefore needed.

By using the formaldehyde-induced fluorescence method (Falck et al., 1962) and its modifications, the indoleamines 5-HT/5-HTP have been demonstrated in the pineal organ of teleosts and other vertebrates (Møller et al., 1979; van Veen et al., 1978; van Veen et al., 1980; for review, see Møller and van Veen, 1981). In the dark-adapted European yellow eel, a blue fluorophore with an ill-defined emission maximum of  $\lambda 430$  nm was found. This spectrum probably indicates the presence of tryptophane (Hartwig and van Veen, unpublished results, Fig. 2).

In the pineal organ of the yellow eel, a diurnal rhythm in 5-HT content is demonstrated (VII). The peak level of the 5-HT content was found at the dark to light transition, but also a high pineal 5-HT content is demonstrated

in the middle of the dark phase. Very little is known about the synthesis, release and/or storage mechanisms within the pineal organ of teleosts partly because the pineal indoleamines in fish are present in an agranular pool. The content of the 5-HT is rather low compared to the amount of 5-HT present in the pineal organs of other vertebrates.

Indoleamines are not only present in the pineal organ of the eel. The paraventricular organ (PVO) with its different subdivisions contains in addition to dopamine also 5-HT/5-HTP formaldehyde-induced fluorescence (VII). Other catecholaminergic nuclei of the diencephalon are the PVO accompanying cells (noradrenalin), the nucleus hypothalami anterior (catecholaminergic) and the recessus preopticus. The pars intermedia of the pituitary receives an inhibitory catecholaminergic innervation of which the exact origin is unknown. Different catecholaminergic nuclei are, however, proposed. The nature of the formaldehyde-induced fluorescence was identified by microspectrofluorometry.

A retinohypothalamic projection (Fig. 4) was demonstrated in the eel using  $\text{Co}^{++}$  iontophoreses (Ekström, unpublished results). This is also reported in other teleosts (cf. Ebbesson and O'Donnel, 1980) and other vertebrates (cf. Oksche and Hartwig, 1975). The function of this projection is unknown.



An indication of an ill-defined photolabile compound in the vicinity of the third ventricle of the eel is also of interest (Hartwig and van Veen, unpublished results). This photolabile compound is, however, not so clearly reproducible as that demonstrated in Phoxinus phoxinus (cf. Oksche and Hartwig, 1979; see also Fig. 5, van Veen and Hartwig, unpublished results).

## Conclusions

From the results of the presented investigations, it can be concluded:

1. The European yellow eel possesses extraretinal and extrapineal photoreception involved in synchronization processes of locomotor activity to environmental lighting.
2. Intact animals synchronize (or mask) their locomotor activity to a photoperiod containing at least  $6 \times 10^{-3} \mu\text{W}/\text{cm}^2$  of green light ( $\lambda$  548 nm,  $\Delta\lambda$  16 nm) and  $10^{-2} \mu\text{W}/\text{cm}^2$  of white light (2750°K)
3. Light of long wavelengths (650-700 nm) penetrates 100-1000 times better into the hypothalamus than light of short (400-450 nm) wavelengths.
4. Blinded and pinealectomized eels synchronize (or mask) their locomotor activity to a similar amount of energy in the light phase of the photoperiod as to which intact eels synchronize (or mask) their activity.
5. The maximum sensitivity range of the encephalic photoreceptor is rather broad but does not include the long wavelengths.

6. The parapineal organ of the eel does not play a major role in the synchronization (or masking) of the locomotor activity to a photoperiod.

7. The parapineal organ of the elver consists of small neurons carrying atypical cilia. These cilia project partly in the intercellular space and partly towards the middle of the organ where desmosome-like junctions are present.

8. The pineal organ displays a diurnal rhythm in 5-HT content in the yellow eel.

9. Monoaminergic nuclei and tracts, involved in rhythmicity, were demonstrated in the diencephalon and telencephalon of the eel.

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## LEGENDS

Fig. 1. One day locomotor activity recordings of a blinded and pinealectomized European minnow Phoxinus phoxinus kept under LD 12:12 with one hour dusk and dawn. Black bar denotes darkness. Actograms showing hourly activity expressed as percentage deviation of mean activity.

- a. recordings from lower photocell showing nocturnal behaviour.
- b. recordings of upper photocell showing diurnal activity
- c. combined recordings of upper and lower photocell showing an apparent arrhythmic behaviour.

Fig. 2. Fluorescence micrograph of the pineal organ of the yellow eel exhibiting blue fluorescence. Note the absence of fluorescence in the surrounding saccus dorsalis.

Fig. 3. Pineal complex of the European minnow.  
c.h. = habenular commissure, III. = third ventricle, star = pineal stalk, arrow = para-pineal organ. Scale marker 20  $\mu$ m. Hor., Azan.

Fig. 4. Transversal section showing retinofugal projection (arrowheads) to the hypothalamus of the eel medial to the fasciculus medialis tracti optici (small arrows). The retinofugal fibers were visualized by means of cobalt iontophoresis. Scale marker 100  $\mu$ m. FDT0, fasciculus dorsomedialis tracti optici III, third ventricle.

Fig. 5. Spectral transmission recording of the diencephalic ventricular wall of a blinded and pinealectomized European minnow Phoxinus phoxinus. Double beam recordings on frozen sections prepared in infrared light using an infrared image convertor.

\* Double beam recordings before irradiation with  $\lambda$  515 nm for 2 min.

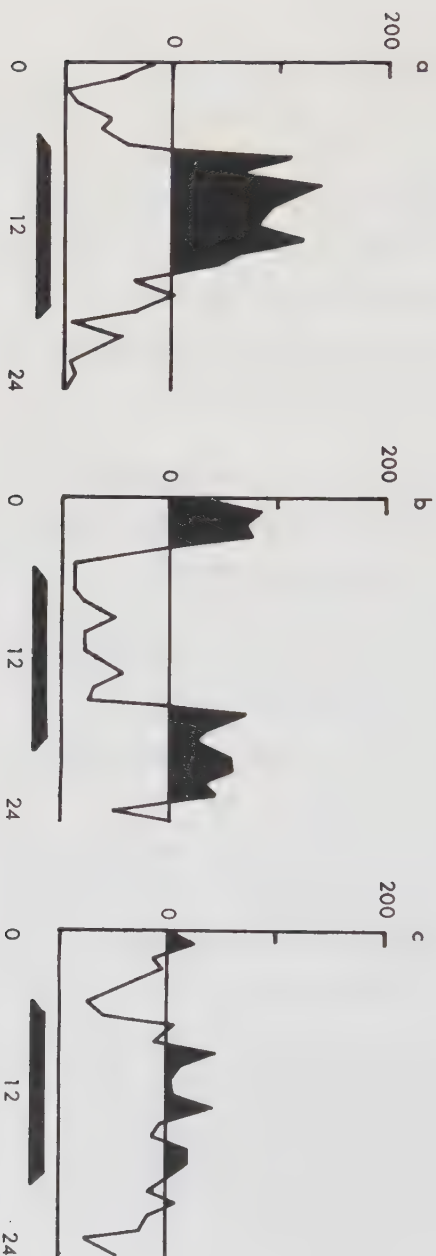
• Double beam recordings after irradiation with  $\lambda$  515 nm for 2 min.

+ Control double beam recording without specimen in sample beam.

Sample beam: ependyma of the third ventricle pre-optic recess area. Reference beam: ventricular cavity.

Note bleaching at wavelengths around 560 nm.

Fig. 1



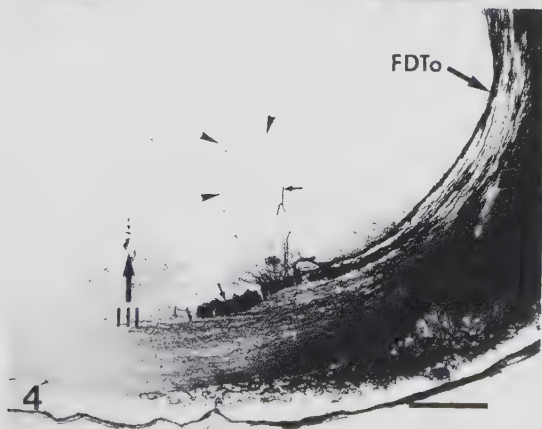
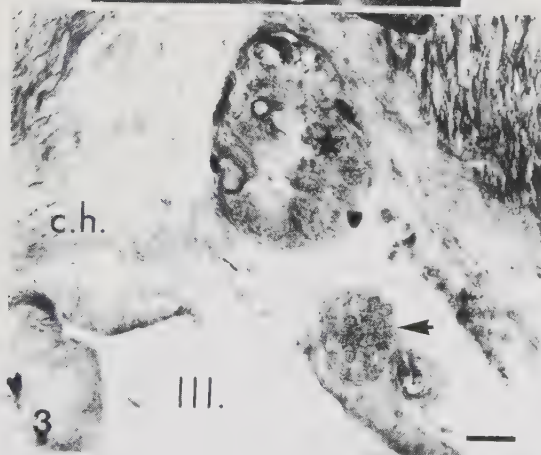
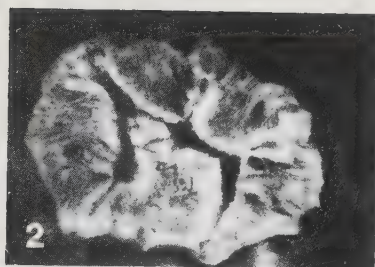
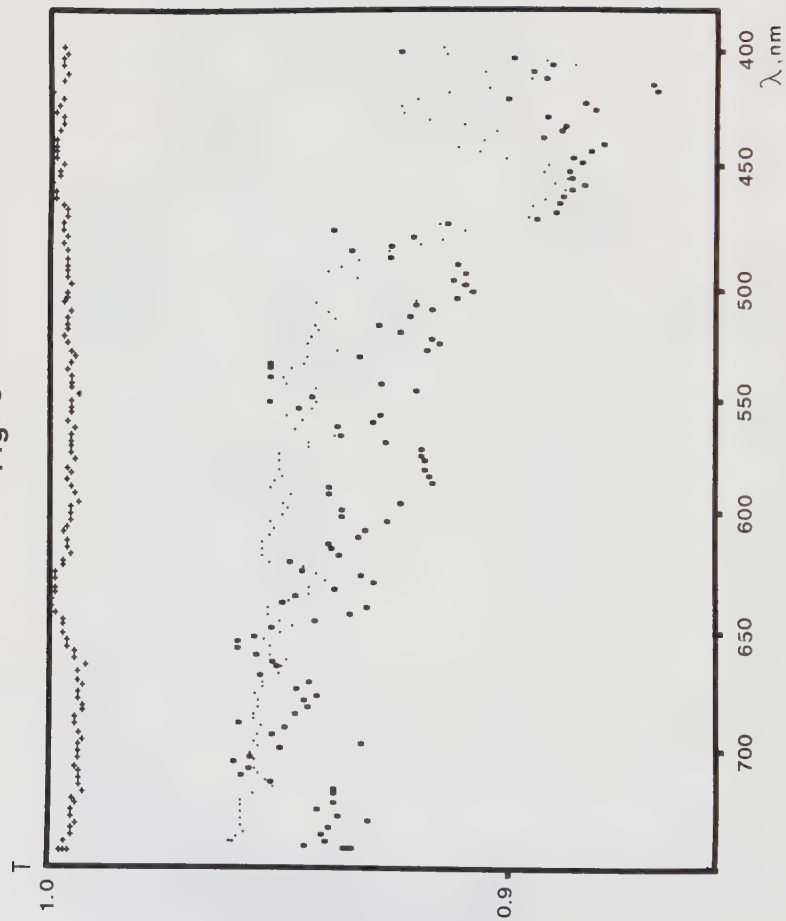


Fig. 5





DIEL RHYTHM IN THE ELVER, ANGUILLA ANGUILLA - TESTING  
A COMPUTERIZED SYSTEM FOR RECORDING BIORHYTHMICS

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## ABSTRACT

An automatic laboratory system designed for monitoring and recording biorhythmic events is described. Interest was focused on controlling the experimental environment. For this purpose, the light regime is adjustable for twilight facilities, intensity and spectral composition, and diel rhythm. Temperature can be regulated in order to maintain constant conditions or produce cyclic fluctuations. With this laboratory system the diel rhythm of the elver showed that these small eels exhibit the same activity pattern as the yellow eel, namely nocturnal behaviour in locomotor activity.

## INTRODUCTION

Studies of the diel rhythm of the eel, Anguilla anguilla, have shown a nocturnal behaviour (Müller 1972, van Veen et al. 1976, Westin and Nyman 1979). van Veen et al. (1976) also showed an arrhythmic behaviour when light was prevented reaching different photoreceptor sites (lateral eyes, pineal organ and encephalon) by means of surgical incisions and covering trials, and a negative phototactic behaviour. Edel (1979) showed shelter behaviour and a tendency to a free running rhythm in the related species Anguilla rostrata, the American eel.

The eel passes through a number of different metamorphic stages (leptocephalus larvae, elver and yellow eel) before it reaches its reproductive stage. This metamorphosis involves profound physiological changes, most dramatically in the change from leptocephalus larvae to elver and from yellow eel to silver eel (the reproductive stage), when significant changes occur in the optical system (for review see Tesch 1973).

In order to demonstrate the diel rhythm in the locomotor activity of the elver, since investigations of the photoreceptive systems of this animal are in progress, we studied this stage of the European eel in the laboratory system described below.

## MATERIAL AND METHODS

Twelve pigmented elvers, Anguilla anguilla L., caught in the river Viskan in southern Sweden, were used. The elvers were kept separately in a light regime of LD 12:12 ( $L = 1 \times 10^{-5} \text{ W cm}^{-2}$ ,  $D < 1 \times 10^{-10} \text{ W cm}^{-2}$ ). After an initial recording period a phase shift (-4 h) was performed after which the recording continued.

### Description of the laboratory system

Because the nature of the experiments to be performed with the system includes constant monitoring and recording of rhythmic events, a computer is constantly on-line. The computer used is a Compucorp 625 Mk II (52k bytes) provided with two disk units for flexible disks (315k bytes/disk), a Real Time Clock and a parallel input-output device (Z80 PIO). The computer is also equipped with a video screen and a 40 column alphanumeric printer. The program language is BASIC with call instructions in ASSEMBLER.

External facilities for the computer include a digital-controlled event detector, which initiates the computer to read the PIO ports at any desired time intervals from 1 to 9999 seconds, and a digital-controlled light intensity regulator (phase-controlled lamp dimmer).

Photocell equipment (modified Visolux ML5) is used for registration of the fish rhythmicity. These photocells were modified in order to penetrate a column of tap water of at least 250 mm, including the glass walls of the tank. It is noteworthy that the photocells can differentiate objects as small as 2 mm in diameter.

The wavelength with which the transmitter operates is greater than 930 nm, which insures that fish will not be able to detect light from the photocells (Schäfer 1964, Müller and Schreiber 1967, Eriksson 1975, van Veen et al. 1976, Eriksson and van Veen 1980). When the photocell beam is interrupted, an event is recorded by the event detector and read by the computer with the previously set time interval. The time interval used in the present study was 20 s. The events are subsequently stored on the flexible disks for later analysis.

Since the natural light-dark variations form the most important Zeitgeber in biorhythmic phenomena, special attention was given to the light facilities of the system.

The light source, in a room without windows, consists of two slide projectors (Zeiss Perkeo 250) provided with 250 W halogen bulbs. These projectors are modified according to the following system. An extra lens system,

containing a focusing lens and a condenser lens, is built in and an iris diaphragm is placed at the focal point in order to modulate the light intensity ( $\text{W cm}^{-2}$ ) without changing the distribution and spectral composition of the light (Fig. 1). This is done to alter the light intensity and to calibrate the two projectors to give an exactly equal amount of light. The projector beam is directed towards the aquaria by means of a mirror. The projectors are enclosed in light-tight metal boxes with openings only for the light beams to pass through. The boxes are ventilated by external fans and the hot air is led away through tubes. The system is provided with twilight facilities controlled by the computer via the light regulator (256 steps). However, the spectral characteristics of the light vary with the fluctuating bulb filament temperature. The spectral composition of the light contains increasing amounts of red components with decreasing light intensities. This thus, resembles the natural conditions where twilight composition also contains a large amount of long waves.

The system is furthermore equipped with Schott double band interference filters and Schott color separation filters (Fig. 2) to permit investigation of the spectral sensitivity of photoreceptors (Zeitgeber receptors) (Hartwig and van Veen 1979). As mentioned above, the projectors provided with iris diaphragms, which enable

modification of the radiated energy. Thus, by measuring the energy per square cm for each wavelength used and by adjusting these measured values to each other by means of the iris diaphragms, it is possible to secure an equal amount of radiant energy over the entire visible spectrum.

Thanks to the various possibilities of regulating the light, the system is also suited for studies related to liminal light perception. Absolute limits for the amount of energy provided depend on measuring facilities. In the present system, a radiometer with the lowest measuring ability of  $10^{-10} \text{ W cm}^{-2}$  is assumed to be adequately sensitive.

The temperature regulation is restricted to the aquaria. All aquaria are provided with running tap water at a constant temperature of  $22 \pm 0.5^\circ\text{C}$  throughout the experiment. However, a cyclic diel temperature rhythm, controlled by the computer, can be provided. The fluctuations of the water temperature under constant conditions can be ignored in connection with Zeitgeber effects, since small temperature differences will randomly occur.

The heating elements are placed in a tank where the water is conditioned to the desired temperature. From this tank the water is pumped into the aquaria. Because of the great capacity of the heating elements, on-off switching of the thermostat is electronically

adjusted in order to avoid tension fall in the electrical circuit, which could disturb the electronic equipment.

Since recirculating water cannot be used in rhythmic research (pheromones), the spill water passes a counter flow heat exchanger (Alfa Laval) to minimize energy loss and expenses (Fig. 3).

## RESULTS AND DISCUSSION

That all organisms express some sort of rhythm (circadian or not) is generally accepted (Aschoff 1960). Attention is most often focused on the detection and recording of these rhythms under laboratory conditions. The environmental conditions in the laboratory are often far from natural for the organisms assayed, which could explain some of the difficulties in obtaining reliable records of their rhythmicity. Working under such conditions will cause problems in evaluating the results of such investigations because of the many cyclic variables involved (radiation, temperature, composition of the respiratory gases, pheromones etc.). Changing one variable might imply that another variable takes over the Zeitgeber function if the unchanged variables cannot be controlled and kept constant, or at least be measured and evaluated.



In the design of the laboratory system described above, efforts were made to control the various environmental variables in order to exclude Zeitgeber information other than the investigated response of a particular environmental Zeitgeber.

Thanks to the high degree of automatization of the laboratory system the animals are disturbed as little as possible during the experiments. It also guarantees a very accurate registration of the biorhythmical events.

As shown by the recording analyses (Fig. 4), the laboratory system functioned satisfactorily. The elvers showed a nocturnal rhythm similar to that of yellow eels (van Veen et al. 1976).

#### Acknowledgements

The authors are indebted to Mr U. Lingärde and Mr L. Lindskog from AB Datakraft and Mr B. Larsson and Mr T. Andersson from Beta Industri Elektronik for their good co-operation and stimulating discussions. Supported by a grant from the Swedish Natural Science Research Council (NFR 2126-100).

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## LEGENDS

- Fig. 1. Optical ray path.  
A halogen bulb, B mirror, C collector lens,  
D condensor lens, E heat absorption filter,  
F spectral selection filter, G iris diaphragm.
- Fig. 2. Spectral characteristics of the light transmitted by some of the filters used in the system (DAL 403-605 Double Band Interference filters and RG 665 Color Separation filter).
- Fig. 3. Running water system.  
A water supply, B counter flow heat exchanger, C water conditioning tank, D heating element (thermostat regulated), E mixer, F air supply, G pump, H aquarium, I drainage tank, J drain.
- Fig. 4. Locomotor activity of the elver. Typical recordings from three consecutive days before (A) and after (B) a four hours' phase shift. y-axis = hourly activity as per cent deviation from the diurnal mean activity. x-axis = hours after start of the experiment (1200). Line under x-axis denotes dark period.

Fig.1

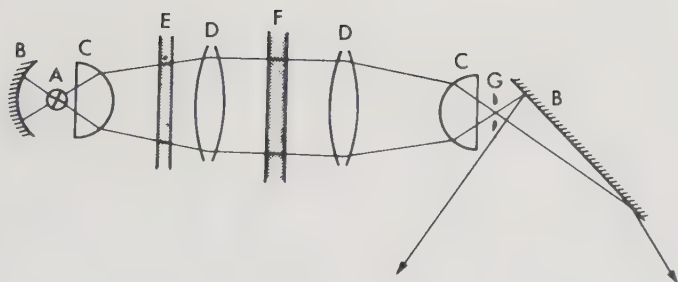


Fig.2

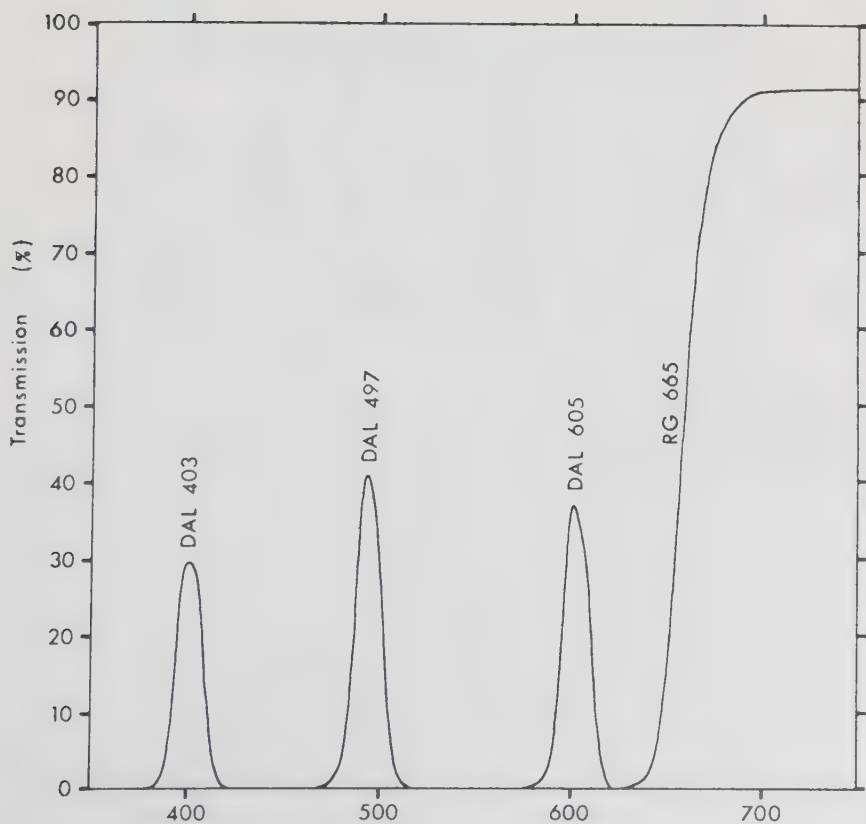


Fig. 3

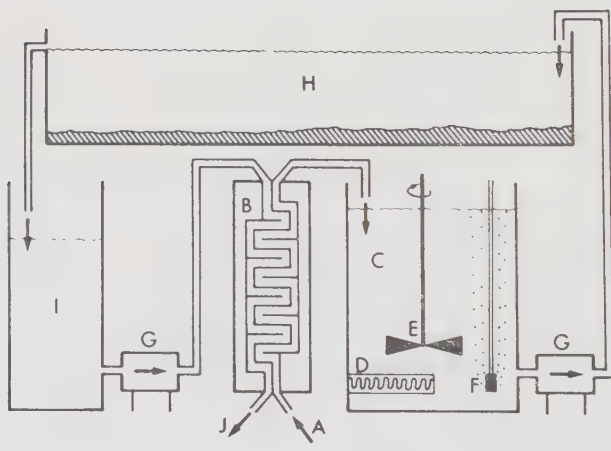
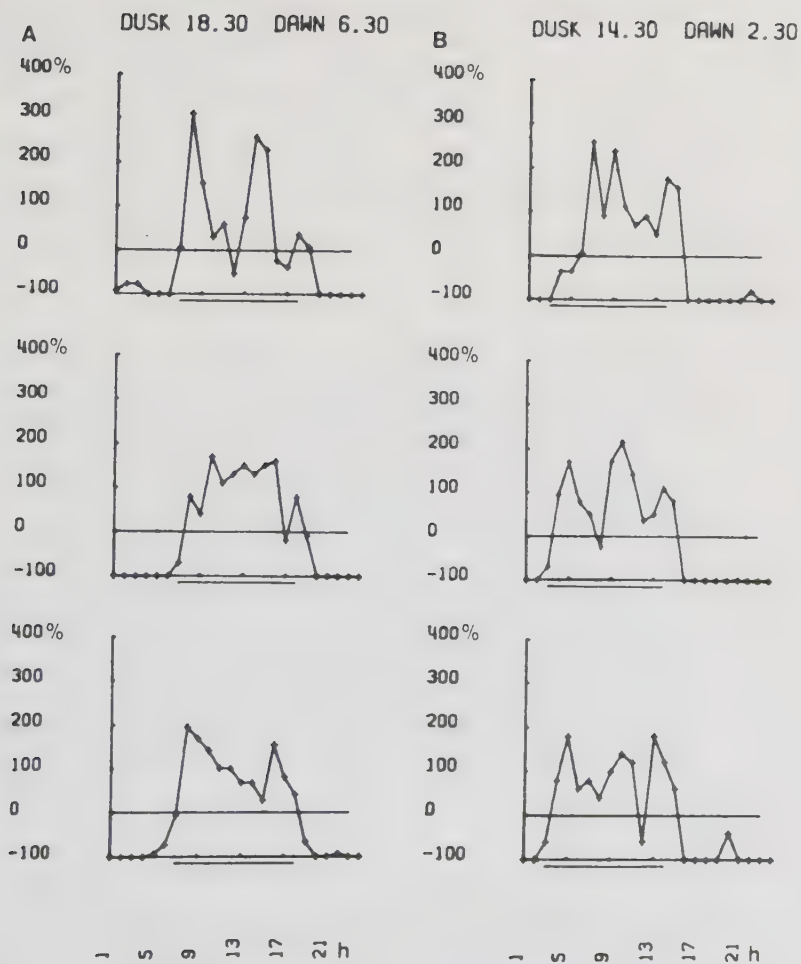


Fig. 4







# Light-dependent Motor Activity and Photonegative Behavior in the Eel (*Anguilla anguilla* L.)

## Evidence for Extraretinal and Extrapineal Photoreception

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**Summary.** Light-dependent motor activity and phototactic behavior was investigated in 1) untreated, 2) blinded, and 3) blinded and pinealectomized eels (*Anguilla anguilla* L.). Neither blinding nor blinding combined with pinealectomy interfered with the observed nocturnal motor activity or the photonegative behavior characteristic for the untreated animals. However, an aluminum foil covering the skull of blinded animals altered the light-dependent motor activity pattern in contrast to blinded animals bearing a transparent plastic foil cover. Blinded animals with an aluminum foil covering the brain case exhibited a motor activity pattern resembling arrhythmicity.

The motor activity pattern of 1) untreated, 2) blinded, as well as 3) blinded and pinealectomized eels followed an inversed light regime with a latency of about two days indicating that in the eel light is the dominating *Zeitgeber* triggering circadian motor activity patterns. The reported findings speak in favor of the existence of photosensitive areas in addition to the lateral eyes and the epiphysis cerebri. From the results of the covering experiments it is clear that these unknown photoreceptor sites must be located in the brain.

## Introduction

It appears to be established that most organisms possess a "biological clock", a mechanism which autonomously controls a number of physiological processes within the organism. The changes in these processes are cyclic, showing a phase of approximately 24 h (circadian rhythm). The circadian rhythm is synchronized with external variables, *Zeitgeber* (Aschoff, 1954). The dominating *Zeitgeber* is usually the natural light-dark change. If the *Zeitgeber* is too weak, or if

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its periodicity lies outside the time schedule where synchronization is possible (Wever, 1972), the organism will show its own endogenous rhythm. Under these conditions some of the investigated organisms react apparently arrhythmically. This has been frequently reported in fish (for review see Eriksson, 1975).

Investigations dealing with rhythmicity of fish have been performed e.g. in the following European species: *Salmo salar* (Kalleberg, 1958), *Salmo trutta* (Müller, 1969), *Cottus gobio* (Andreasson, 1969), *Cottus poecilopus* (Müller, 1970), *Myoxocephalus quadricornis* (Westin, 1971), *Salvelinus fontinalis* (Eriksson, 1972a, b), *Anguilla anguilla* (Müller, 1972), *Lota lota* (Müller, 1973), and *Perca fluviatilis* (Eriksson, 1975). For further references see Schwassmann (1971), and Richardsson and McCleave (1974). These experimental findings clearly demonstrated that light-dark cycles act as the dominating *Zeitgeber* in fish.

Motor activity recordings performed in the European eel, *Anguilla anguilla*, at the arctic circle (Müller, 1972) demonstrated that this species is nocturnal (night active), and that the eel rests during the winter period, November–May, due to low water temperature (below 4 °C). Nocturnal behavior has also been reported by Bohun and Winn (1969) in the American eel, *Anguilla rostrata*.

The water temperature determines the level of activity whereas the day length determines the duration of the diel (circadian) activity period (Müller, 1972). Since light-dark changes seem to be the dominating *Zeitgeber* triggering daily events, considerable effort has been made to localize photosensitive areas other than the lateral eyes, which were—as originally observed in birds (cf. Menaker, 1968)—not of exclusive importance in this connection.

It has been known for a number of years that functioning photoreceptors are present in the pineal organ (epiphysis cerebri) of various fish (electrophysiological studies: Dodt, 1963; for review see Dodt, 1973; Hamasaki and Eder, 1975; behavioral studies: de la Motte, 1964; morphological studies: for review see Oksche and Kirschstein, 1971). Rudeberg (1971) presented in an electron microscopic analysis of the pineal organ of *Anguilla anguilla* strong evidence for the existence of lamellar photoreceptor outer segments.

However, in blinded and pinealectomized European minnows, *Phoxinus phoxinus*, an additional light sensitive region of the diencephalon exists in the vicinity of the third ventricle. This photosensitive area is able to mediate color changes (von Frisch, 1911) and light dependent conditioned reflexes (Scharrer, 1928; for further considerations of the problem of extraretinal "deep diencephalic photoreceptors", see Oksche and Hartwig, 1975). In submammalian vertebrates extraretinal and extrapineal photoreceptors might be involved in the photoperiodic control of circadian and circannual rhythms (for review, see Menaker, 1971).

The present investigation in the eel, *Anguilla anguilla*, gives additional evidence for the existence of photoreceptor sites, other than the lateral eyes and the pineal organ, localized in the brain, presumedly in the diencephalon.

## Material and Methods

Eels (*Anguilla anguilla* L.) captured in the Öresund near Malmö, and with an average body weight of 60 g, were used in this experiment.

The phototactic behavior of intact, bilaterally ophthalmectomized (bulbectomized) as well as that of bilaterally ophthalmectomized and pinealectomized eels kept in rectangular tanks provided with running tap water under DD (constant darkness) conditions was analyzed using a focussed light beam of 1 cm diameter. The light source was a tungsten filament (6 V, 30 W). The electric tension used was 4.5 V. The light beam was produced by a collector lens including a heat absorption filter, a variable diaphragm and a projecting lens. It was possible to illuminate continuously various body regions of the free swimming animals.

In a second group of eels the motor activity was recorded according to a method described by Müller and Schreiber (1967). Each animal was placed in an opaque glass-fiber tank, shaped as a circular channel, provided with running tap water and a photocell unit. The tanks were covered with plexi-glass to prevent escape of the animals. One third of the tank surface was covered with aluminum foil. The light source of the photocell unit was in the infra-red range ( $\lambda > 850$  nm). Light beam interruptions were recorded by an event printer (Elmeg, response delay: 5s). The data were assembled in histograms (Fig. 1-5) in which the columns show the accumulated recordings over three hour-intervals assembled over a period of ten days.

A light schedule with rectangular light-dark changes was arranged by means of a timer, with a phase of 12 h light and 12 h dark (LD 12:12). Philips TL fluorescent daylight tubes 40 W 55 served as light sources. The light intensity measured on the water surface was 680 lux, beneath the covered part of the tank the intensity was 80 lux. The water temperature was kept at approximately 16 °C, but variations of  $\pm 2$  °C could not be eliminated.

Some days before the commencement of activity recordings, animals were placed under the experimental conditions. Four groups of animals were studied (Tables 1 and 2):

Group 1: Animals recorded before and after two types of subsequently performed operative incisions (bilateral ophthalmectomy and subsequent pinealectomy).

Group 2: Animals provided with an opaque cover over the brain case (see technique below); some of the animals were bilaterally ophthalmectomized before receiving the cover.

Group 3: Sham operated controls.

*Surgical Technique.* The animals were anesthetized in a solution of Urethane in water (35 g/l) until the gill movements stopped. Thereafter the animals were wrapped in a wet paper towel to prevent desiccation.

*Ophthalmectomy.* This operation was performed in two different ways, depending on the consecutive treatment. In animals which were to be subsequently pinealectomized, a superficial cut was placed over the orbita, whereafter the eye could be removed after rotating it and cutting off the muscles and finally the optic nerve. The second method was used for the covering trials. Following a median cut through the skin of the dorsal head the dorsal skull bones and the orbitae were exposed by dissecting the covering muscles. Subsequently, the external eye muscles and the optic nerves could be transected and the eye bulbs removed.

*Pinealectomy.* This procedure was performed only after bilateral ophthalmectomy of type one (see above). After dissecting the covering muscles a piece of bone overlying the pineal region was cut out with a dental drill. The saccus dorsalis containing the pineal vesicle and a part of the pineal stalk was then visible through the meninx. The meninx was opened and the saccus dorsalis together with the pineal vesicle and a part of the pineal stalk removed. The piece of bone was replaced and the skin sutured with silk thread.

*Covering Trials.* After ophthalmectomy, small balls of aluminum foil were placed in the empty orbit, and as large a part of the skull as possible was covered with a piece of the same material. The muscles and skin were replaced and the covering was fixed by suturing the skin with silk threads.

The operations, which took approximately half an hour each, were tolerated well by the animals. They recovered soon after being placed back in their respective tanks. In order to assess the size of the incision, the animals were killed by decapitation at the end of the experiment. The brains were dissected out, fixed in buffered glutaraldehyde, subsequently split midsagittally and processed for scanning electron microscopy (critical point drying in Freon and coating with a layer of gold-palladium).

## Results

### *Phototactic Behavior*

Six untreated eels kept in darkness (DD) showed a characteristic backward swimming behavior if a focussed light beam of 1 cm diameter illuminated the head. The response time (beginning of illumination to motoric response) was between 3 and 6 s. After bilateral ophthalmectomy the response time ranged between 20–30 s. Subsequently performed pinealectomy increased the response time to 30–80 s. If an animal did not respond within 100 s after the initiation of illumination of the head region, this event was recorded as a negative response. The number of animals not reacting to the light stimulus was higher in the first postoperative days after ophthalmectomy and subsequently performed pinealectomy. After a recovery period of four days all animals showed a clear

**Table 1.** *Anguilla anguilla*, motor activity rhythm. Experimental conditions and results of group 1

| Animal No. | Type of incision              | Length of registration | Light regime        | Rhythm   |
|------------|-------------------------------|------------------------|---------------------|----------|
| 1–2        | intact                        | 14 days                | LD 12:12            | rhythmic |
| 3–4        | intact                        | 44 days                | LD 12:12            | rhythmic |
| 1–2        | ophthalmectomy                | 30 days                | LD 12:12            | rhythmic |
| 3–4        | ophthalmectomy                | 20 days                | LD 12:12            | rhythmic |
| 1–2        | ophthalmectomy                | 20 days                | LD 12:12 (inversed) | rhythmic |
| 1–4        | ophthalmectomy + pinealectomy | 20 days                | LD 12:12            | rhythmic |
| 3–4        | ophthalmectomy + pinealectomy | 20 days                | LD 12:12 (inversed) | rhythmic |

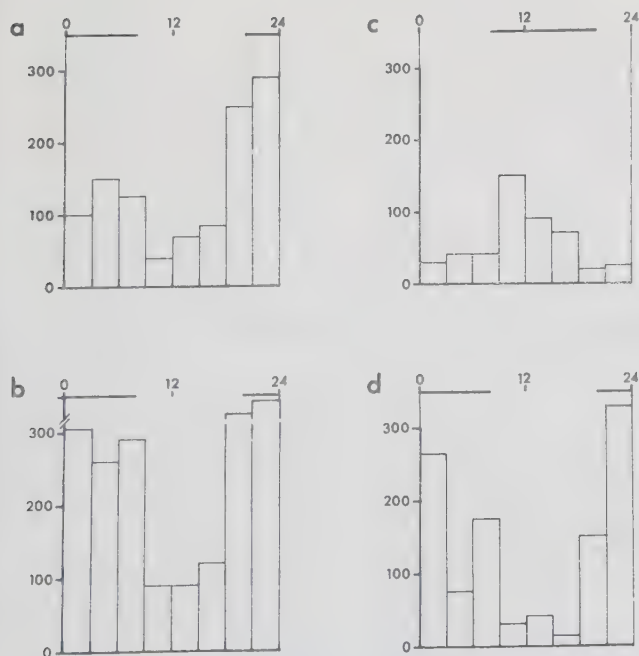
**Table 2.** *Anguilla anguilla*, motor activity rhythm. Experimental conditions and results of group 2

| Animal No. | Type of incision                          | Length of registration | Light regime | Rhythm     |
|------------|-------------------------------------------|------------------------|--------------|------------|
| 5–12       | intact                                    | 14 days                | LD 12:12     | rhythmic   |
| 5–6        | opaque cover intact eyes                  | 20 days                | LD 12:12     | rhythmic   |
| 7–10       | bilateral ophthalmectomy and opaque cover | 20 days                | LD 12:12     | arrhythmic |
| 11–12      | ophthalmectomy and transparent cover      | 20 days                | LD 12:12     | rhythmic   |

photonegative behavior. Neither in untreated nor in operated animals did illumination of other body areas result in the reported characteristic backward swimming behavior or in any other observable short time motoric response. In untreated and in operated animals the response time showed a clear dependence on the light intensities applied. It is worthwhile to mention that a blinded and pinealectomized animal after a survival period of 9 months also showed the described photonegative response.

### *Light Dependent Motor Activity*

The motor activity of 12 eels was investigated under various conditions (Tables 1 and 2).



**Fig. 1a-d.** Motor activity of one individual eel under experimental conditions shown in Table 1. **a** Untreated animal. **b** after bilateral ophthalmectomy. **c** bilaterally ophthalmectomized animal, inverted photoperiod. **d** bilaterally ophthalmectomized and pinealectomized animal, conventional photoperiod

All trials began with the activity recording of untreated animals. In this experimental stage all eels showed a nocturnal behavior (Figs. 1a–5a). Bilateral ophthalmectomy and bilateral ophthalmectomy combined with pinealectomy did not affect this behavior (Figs. 1b, d, 2b, c). As is evident from the histograms (Fig. 1c, 2d), all animals showed an adjustment of their motor activity behavior corresponding to a reversal of the photoperiod. Animals bearing an opaque cover over the bony skull also demonstrated nocturnal locomotor activity as long as the visual system was not affected (Fig. 3). However, in animals bearing an opaque cover, bilateral ophthalmectomy resulted in a completely different pattern of motoric behavior. The animals no longer showed a rhythm adjusted to the given light schedule; apparently their motor activity was arrhythmic (Fig. 4). In sham-operated control animals, bearing a transparent plastic foil instead of an opaque aluminum foil covering the brain case, bilateral ophthalmectomy did not influence the nocturnal activity pattern. Reversal of the photoperiod induced a reversal of the motoric activity pattern.

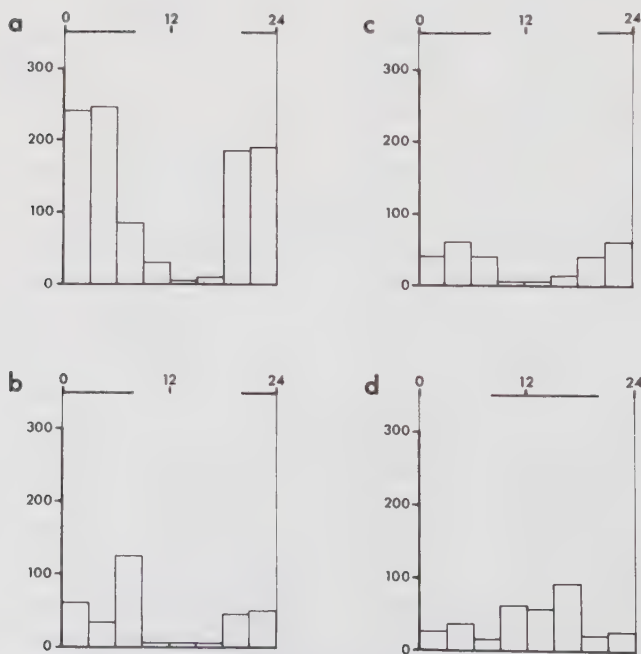
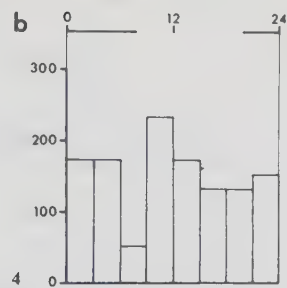
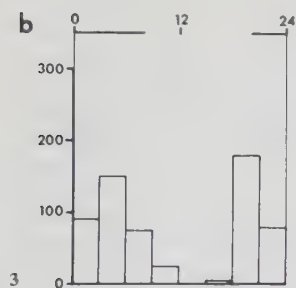
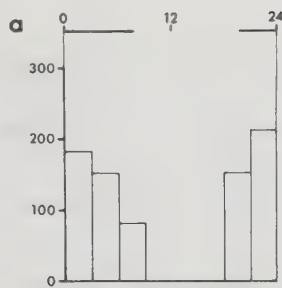
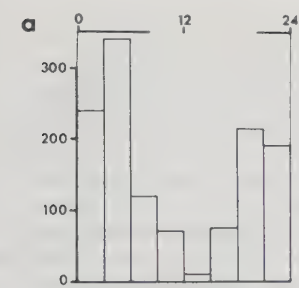
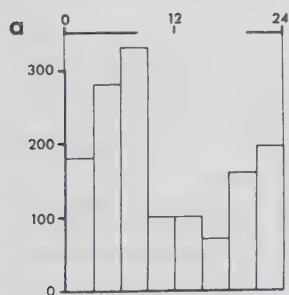


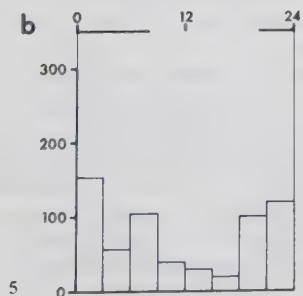
Fig. 2a–d. Motor activity of one individual eel under experimental conditions shown in Table 1. a Untreated animal, b after bilateral ophthalmectomy, c bilaterally ophthalmectomized animal after pinealectomy, d bilaterally ophthalmectomized and pinealectomized animal, inversed photoperiod



**Fig. 3a and b.** Motor activity of one individual eel under experimental conditions shown in Table 2.  
**a** Untreated animal,  
**b** after capping the bony skull with an aluminum foil



**Fig. 4a and b.** Motor activity of one individual eel under experimental conditions shown in Table 2.  
**a** Untreated animal,  
**b** after bilateral ophthalmectomy and after capping the bony skull with aluminum foil. Note the apparent arrhythmicity of motor activity



**Fig. 5a and b.** Motor activity of one individual eel under experimental conditions shown in Table 2.  
**a** Untreated animal,  
**b** after bilateral ophthalmectomy and after capping the bony skull with transparent plastic foil. Note the synchronization of motoric activity with the applied photoperiod



## Discussion

Müller (1972) demonstrated that under natural conditions light is the dominating *Zeitgeber* triggering the motor activity of the European eel, *Anguilla anguilla*, and that the animal is essentially nocturnal. In the present investigation the *Zeitgeber* effect of light was tested under artificial conditions.

In order to supply an effective *Zeitgeber* an experimental photoperiod was chosen LD (12:12) and the light intensity adjusted to 680 lux. In the present study it was not possible to record other photometric values than lux. Photoreceptor activity depends on the number of photons absorbed per time unit. Therefore, photometric values based on "standard eye" sensitivity give only very limited information on the number of photons reaching any retinal or extraretinal photoreceptor, even if the spectral energy distribution of a light source is known. Nevertheless the reported lux values give a rough estimate of the strength of the applied *Zeitgeber*. The unavoidable fluctuations in water temperature of about 2 °C could in fact act as an additional *Zeitgeber* in the operated animals due to oversensibilization or compensation (see Hoffmann, 1968). However, water temperature fluctuations as well as changes in oxygen content of the running tap water could be excluded as dominating *Zeitgeber* by the experimental reversal of the photoperiod. Motor activity patterns followed the reversal of the photoperiod within a few days as long as light reached retinal or extraretinal photoreceptors.

The fact that the animals refused food during their captivity was a positive factor in this investigation because it excluded the possibility of time related information (feeding).

The topographical arrangement and the small size of the pineal organ make it impossible to remove pineal tissue without damaging the surrounding dorsal sac epithelium. Therefore, in "pinelectomy" the saccus dorsalis and probably also other epithelial structures of the diencephalic and telencephalic roof were damaged or removed together with the pineal vesicle and parts of the pineal stalk. Neuroendocrinologists have proposed that a hormone (indolamine) is released from the pineal organ which might influence circadian rhythms (for review see Wurtmann et al., 1968; Hamasaki and Eder, 1975; studies in lower vertebrates see Bagnara and Hadley, 1970; Ueck, 1974). Even in bilaterally blinded animals the removal of the epiphysis cerebri including the dorsal sac and adjacent epithelial structures did not alter the adjustment of motor activity to a given light schedule. However, this finding does not exclude a cyclic release of biological active substances from the epiphysis cerebri.

Some unspecific postoperative effects might have influenced the diel rhythm, but these appeared to be of short duration. One of these effects might have mediated a high, apparently arrhythmic locomotor activity during the first two postoperative days. A long lasting effect was a highly aggressive behavior in blinded animals.

The reported findings in blinded and pinealectomized eels speak in favor of the existence of a photosensitive system, different from the lateral eyes and the pineal organ, which – in the present experiment – might act as a receptor for the *Zeitgeber*.



The covering experiments were designed to localize the unknown photosensitive area (s). In these animals both eyes were removed and a piece of aluminum foil was placed under the skin and the muscles covering the bony skull dorsally and bilaterally. In sham operated control animals instead of a piece of aluminum foil, a transparent plastic foil was inserted covering the skull. The latter animals showed a normal locomotor rhythm, as did animals bearing an aluminum foil cover but with intact visual apparatus. These results indicate that blinded eels, after preventing light from penetrating into the brain, are unable to receive information necessary to adjust their motor activity to the *Zeitgeber*. At the same time this result excludes dermal photoreception playing a role in triggering motoric behavior. Dermal photoreception at the trunk and the tail was suggested by de la Motte (1964). We found, however, no evidence for the existence of these receptor sites.

This conclusion is further supported by the reported photonegative behavior seen in blinded and pinealectomized eels. The light-induced characteristic backward swimming activity might be a part of a general avoidance behavior of eels. However, only when using a focussed light beam projecting to the head was it possible to force the animal to continue with the characteristic backward moving activity.

Since illumination of body areas other than the head did not result in a photonegative response, the extraretinal and extrapineal photosensitive area must be localized in the head region. A small light beam projected to the head region of the eel is spread by penetrating skin, the muscles and the bone. Therefore, only light beams of 1 cm diameter were employed. However, von Frisch (1911) and Scharrer (1928) using microsurgery techniques in blinded and pinealectomized European minnows, *Phoxinus phoxinus*, have been able to localize a photosensitive area in the wall of the third ventricle. Von Frisch (1911) speculated on photosensitive elements situated between the ependymal cells covering the third ventricle. In *Phoxinus phoxinus*, Hartwig (cf. Oksche and Hartwig, 1975) was able to demonstrate a circumscribed ependymal area of the third ventricle containing a photosensitive compound. This region is rich in bulbous cilia of the 9+0 type which resemble the so-called "rudimentary" photoreceptor outer segments observed in the pineal stalk. Further experimental studies are necessary to prove whether these structures are functioning photoreceptors.

In the present experiment even an analysis of single day motoric activity recordings did not result in complete data concerning a free running rhythm in eels blinded and covered with an aluminum foil. In fish it is difficult to demonstrate free running rhythms (cf. Eriksson, 1975). One may suggest that, in the eel under experimental conditions, important ecological demands might not be fulfilled. In addition to some possible shortcomings of the applied recording unit of this experiment (only one photocell per tank) this might also explain the apparent arrhythmicity observed instead of an expected free running rhythm.

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THRESHOLD FOR SYNCHRONIZATION OF LOCOMOTOR ACTIVITY  
TO VISIBLE RADIATION IN THE EEL (Anguilla anguilla L.)

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## ABSTRACT

Locomotor activity of twelve small European yellow eels, exhibiting a nocturnal pattern of behaviour, was recorded in a computerized laboratory system. White light with an energy content of  $20 \mu\text{W}/\text{cm}^2$  in the light phase of the day synchronized the locomotor activity of all twelve eels used to the applied LD regime (LD 12:12 with a dusk and dawn period of 30 minutes). During a following period of constant darkness ( $D < 10^{-7} \mu\text{W}/\text{cm}^2$ ; threshold of used radiometer) the eels lost this synchronized activity pattern and their diel locomotor activity appeared to be arrhythmic. When the animals were exposed to an LD regime again, the light phase was shifted  $180^\circ$ . In order to find the energy threshold for synchronization of the locomotor activity to an LD regime, the energy in the light phase was gradually raised. The following levels were used:  $0.02 \mu\text{W}/\text{cm}^2$ ,  $0.05 \mu\text{W}/\text{cm}^2$  and  $0.09 \mu\text{W}/\text{cm}^2$ . An increasing number of eels synchronized their locomotor activity to the LD regime, from four at the low energy level to nine at the high level. When light energy of a specified narrow bandwidth ( $548 \text{ nm } \Delta \lambda = 16 \text{ nm}$ ) was used, one eel out of the twelve synchronized to an energy influx of  $0.004 \mu\text{W}/\text{cm}^2$ , equivalent to approx.  $11 \times 10^9$  quanta/s  $\text{cm}^2$ . An increase of the energy influx to  $0.006 \mu\text{W}/\text{cm}^2$  or approx.  $16 \times 10^9$  quanta/s  $\text{cm}^2$  synchronized six out of the twelve. Subjected

to an energy influx of longer (665-700 nm/0.009  $\mu\text{W}/\text{cm}^2$  approx.  $30 \times 10^9$  quanta/s  $\text{cm}^2$ ) or shorter (403 nm/0.005  $\mu\text{W}/\text{cm}^2$  approx.  $10 \times 10^9$  quanta/s  $\text{cm}^2$ ) wavelengths the animals failed to synchronize. According to these findings the threshold for synchronization to radiation of 548 nm was estimated to be in the order of 0.006  $\mu\text{W}/\text{cm}^2$  and to white light 0.02  $\mu\text{W}/\text{cm}^2$ .

## INTRODUCTION

The European eel, Anguilla anguilla, shows a synchronization of daily and yearly locomotor activity to environmental lighting (Müller 1972, van Veen, Hartwig and Müller 1976, Westin and Nyman 1979), exhibiting a nocturnal pattern of behaviour. As in other investigated nocturnal fish (Hobson 1972, Munz and McFarland 1973, Eriksson 1978, Eriksson and van Veen 1980) the eel displays a photonegative response, i.e., it avoids strong light by hiding.

The closely related American eel, Anguilla rostrata, exhibits a pattern of locomotor activity similar to that of the European eel (Bohun and Winn 1966, Edel 1979). Edel (1979) also demonstrated a tendency to a free-running rhythm in the American silver eel kept in constant conditions.

Thus, light forms an important Zeitgeber in synchronizing locomotor activity in the eel. Therefore, an understanding of the photoreception mechanisms, their sensitivity range and other adaptations is of great importance. The retinal



photoreceptors of the European yellow eel, which mainly lives in freshwater, contain photopigments with  $\lambda_{\max}$  at 501 and at 523 nm (Gordon et al. 1978). In addition to retinal photoreceptors, the European eel also has pineal photoreceptors (Oksche and Vaupel-von Harnack 1965, Rüdeberg, 1971). Pineal photoreceptors are functional units of light perception in fish (for reviews, see: Dodt, 1973, Hamasaki and Eder 1977). Furthermore, a possibility of dermal photoreception in the eel has been claimed by de la Motte (1964). Extraretinal and extrapineal encephalic photoreception in the European yellow eel, synchronizing locomotor activity, was demonstrated by van Veen et al. (1976).

The aim of the present investigation was to study the energy threshold for synchronization of locomotor activity to visible radiation in the European yellow eel.

#### MATERIAL AND METHODS

Twelve small yellow eels (Anguilla anguilla L.), supplied from an eel culture plant, were used in the present study. The animals were kept singly in tanks containing 2 l of aerated running tap water constant in temperature (23.5°C with randomly distributed changes of  $\pm 0.5^\circ$ ). Each tank was provided with a sandy bottom and hiding places (clam shells and/or small stones).

In these tanks, in which the locomotor activity of the animals was to be recorded, the animals were kept for more than 5 weeks prior to the investigation in order to adapt to the laboratory conditions. The animals were fed a surplus of Tubifex at the commencement of the experiments. As the Tubifex thrived in the sandy bottom, food was available during the entire experiment.

The locomotor activity of the eels was recorded by a computerized laboratory equipment provided with photocell units (van Veen, Andersson and Nilsson 1981). Table 1 shows the experiment schedule, which was followed in order to attain light energy thresholds for the synchronization of locomotor activity to a given light-dark regime. The applied light regimes included a dusk and dawn period of 0.5 h. Underwater light intensities were recorded with a radiometer. Intensities of white light and light with narrow bandwidth were changed by a phase-controlled lamp dimmer and a diaphragm at the focal point of the lamp. Light quality was modified by double band interference filters (at 403 nm and at 548 nm;  $\Delta\lambda = 16$  nm) or by a long-pass cut-off filter in combination with a short-pass cut-on filter ( $\lambda = 665-700$  nm) (van Veen et al. 1981).

Quanta of different wavelengths consist of different amounts of energy. Therefore, the energy value of the long ( $\lambda 665-700$  nm) and of the short ( $\lambda 403$  nm) wavelengths were corrected to each other to contain approximately equal amounts of quanta.

Animals were considered as synchronized with the light-dark cycle when the average amount of nocturnal activity exceeded the diurnal activity by at least 50%, and if in addition, clear changes in activity repeatedly coincided with the dark-to-light or light-to-dark transitions.

The colour temperature of the light was measured with a PROFISIX-photometer (Gossen) provided with a PROFI-color (Gossen) accessory device.

## RESULTS

LD 12:12 conditions - white light  $20 \mu\text{W}/\text{cm}^2$  (colour temp. approx.  $3400^\circ\text{K}$ )

When subjected to these LD conditions, all twelve animals showed a typical nocturnal behaviour. Several of the eels ceased their activity 1-2 hours before the lights turned on (Fig. 1a). In most cases the activity started again in the dusk period.

Constant conditions - D:D - less than  $10^{-7} \mu\text{W}/\text{cm}^2$

When offered constant conditions, most animals displayed an apparent arrhythmic pattern of locomotor activity (Fig. 1b).

LD 12:12 conditions - white light  $0.02 \mu\text{W}/\text{cm}^2$  - phase shift of  $180^\circ$  compared to the light regime prior to DD conditions (colour temp. approx.  $2750^\circ\text{K}$ )

Four out of the twelve eels synchronized their locomotor activity according to this Zeitgeber regime. These animals

displayed a nocturnal behaviour (Fig. 1c). However, the amount of activity during the light phase of the day was elevated in comparison to a stronger Zeitgeber regime (Fig. 1a).

LD 12:12 conditions - white light  $0.05 \mu\text{W}/\text{cm}^2$  (colour temp. approx.  $2750^\circ\text{K}$ )

During this light/dark regime six out of the twelve eels showed synchronization of their locomotor activities (Fig. 1d). The displayed synchronized activity patterns were similar to those obtained during the previous light regime.

LD 12:12 conditions - white light  $0.09 \mu\text{W}/\text{cm}^2$  (colour temp. approx.  $2750^\circ\text{K}$ )

Nine out of the twelve eels were synchronized and showed a nocturnal behaviour (Fig. 1e). Their activity patterns were more distinct than those obtained during the weaker Zeitgeber regimes and resembled more the well-defined activity patterns achieved during the strongest Zeitgeber regime, i.e., the activity during the light phase of the day was less pronounced.

LD 12:12 conditions - 548 nm  $0.004 \mu\text{W}/\text{cm}^2$  (approx.  $11 \times 10^9$  quanta/s  $\text{cm}^2$ ) - phase shift of  $30^\circ$  compared to prior light regime

Only one out of the twelve eels showed synchronized nocturnal locomotor activity under this light/dark regime (Fig. 1f). This animal also followed the 2 hours phase shift provided.

LD 12:12 conditions - 548 nm  $0.006 \mu\text{W}/\text{cm}^2$  (approx.  $16 \times 10^9$  quanta/s  $\text{cm}^2$ )

Six out of the twelve eels synchronized with this Zeitgeber regime, showing a nocturnal behaviour (Fig. 1g).

LD 12:12 conditions - 665-700 nm deep red light  $0.009 \mu\text{W}/\text{cm}^2$  (approx.  $30 \times 10^9$  quanta/s  $\text{cm}^2$ )

None of the animals subjected to this light/dark regime showed synchronized locomotor activity (Fig. 1h).

LD 12:12 conditions - 403 nm  $0.005 \mu\text{W}/\text{cm}^2$  (approx.  $10 \times 10^9$  quanta/s  $\text{cm}^2$ )

None of the animals synchronized with the offered light/dark regime (Fig. 1i).

As shown above, with increasing light intensities an increasing number of eels synchronized their locomotor activity to the LD regime (Fig. 2).

The results are summarized in Table 1. Regarded as threshold intensities were for white light  $0.02 \mu\text{W}/\text{cm}^2$  (30% of the animals synchronized) and for radiation of 548 nm  $0.006 \mu\text{W}/\text{cm}^2$  (50% of the animals synchronized).

#### DISCUSSION

This investigation shows that the European yellow eel, in order to synchronize its locomotor activity to a given

light/dark regime, needs visible radiation energy (548 nm  $\Delta\lambda$  16 nm) in the order of  $0.006 \mu\text{W}/\text{cm}^2$  (equivalent to  $16 \times 10^9$  quanta/s  $\text{cm}^2$ ). Radiation of this wavelength (548 nm) is absorbed by the retinal photopigments to about 85% (Carlisle and Denton 1959). Thus, the radiation energy applied was concentrated to an area of the spectrum known to be nearly "optimal" for the eel retina.

The higher energy threshold obtained when white light was used is, of course, expected, especially as the characteristics of the lamp increased in the red components of the light at lower light intensities.

The locomotor activity pattern of eels kept in constant conditions showed a rapid dissociation. In some cases a persistent rhythm was apparent. However, in this context this was not further analysed.

Entrainment from the arrhythmic, or free-running, state to the LD regime always occurred within one or two days. Naturally, it is possible that this quick response to a Zeitgeber is not entrainment and synchronization of the endogenous rhythm but only a masking effect. This, however, is of no immediate importance to the results of this investigation since photoreception is also necessary for masking effects.

This study was performed to find the threshold for synchronization of locomotor activity to visible radiation. Therefore, the experimental period of white light at the lowest intensity was longer than the following stronger intensities. When changing wavelength, a small phase shift was performed in order to distinguish continued synchronization to the given LD regime from eventually occurring after-effects.

Comparisons between the locomotor activity patterns of eels kept in the weaker Zeitgeber regimes and in the initial strong Zeitgeber regime reveal that the amount of day activity increases as light intensity decreases. Weaker light-to-dark transitions (dusk and dawn) as well as the low amount of light energy during the light phase form a weaker stimulus in the phototactic behaviour of the eel. This results in a less pronounced shelter behaviour (visual observations), which might lead to an increased amount of photocell beam interruptions by minor movements of the animals. However, the observed rise in day activity could also be explained by an inherited dualism in locomotor activity pattern as reported in other fish (Müller 1970, 1973, 1978, Eriksson 1975, 1978). This dualism expresses itself as a phase shift in the daily locomotor activity. In nature this is a seasonal phenomenon, but extreme Zeitgeber conditions can also trigger this phase

shift (Müller 1978, Eriksson 1978). The shift can occur rather suddenly or, as demonstrated in the brown bullhead (Eriksson 1978, Eriksson and van Veen 1980), can start as a rise of activity in the light phase.

In the present investigation, conditions to promote a completed change in behaviour pattern were virtually absent. When the light intensities provided were below the synchronization threshold, one can assume that the animals perceived this situation as a constant condition. However, when the Zeitgeber was perceived and the eels entrained, the strength of the Zeitgeber was successively increased within a short period of time, reaching values above the threshold intensities. This in turn had a stabilizing effect on synchronization and a lower amount of day activity was noticed.

The natural environment of the European yellow eel provides a very broad spectrum of lighting conditions. The Zeitgeber regimes used in this investigation scarcely differ from some of these conditions. In some freshwater environments the fluctuations in daily lighting must be even less pronounced. It is therefore surprising that just one eel out of the twelve was able to synchronize to the light/dark regime, with nocturnal or diurnal phasing of its locomotor activity when light of 548 nm and intensity of  $0.004 \mu\text{W}/\text{cm}^2$  was used. Such an intensity in that spectral area must



lay far above the sensitivity threshold of the retina if one considers the measurements of spectral sensitivity of the retina done on the closely related American eel (Gordon, Shapley and Kaplan 1978). This observation indicates extraretinal photoreception, which is known to synchronize locomotor activity in the eel (van Veen et al. 1976) as well as in other fish (Eriksson 1972, Siegmund and Wolff 1972), in amphibians (Adler 1976), in reptiles (Underwood and Menaker 1976) and in birds (Gwinner, Turek and Smith 1971, Menaker and Underwood 1976).

Energy threshold studies on extraretinal encephalic photoreception in Phoxinus have shown that this fish reacts behaviourally to a light stimulus of  $10^8$  quanta/s  $\text{cm}^2$ , when light of the most efficient wavelength was used (about 530 nm) (de la Motte 1964). Thus, it was found in the eel that the energy threshold of  $16 \times 10^9$  quanta/s  $\text{cm}^2$  for synchronization to a light/dark regime does not, from the energy point of view, exclude the possibility of extraretinal encephalic perception. Considering that Phoxinus, in contrast to the eel, is provided with a specialization in the skull (pineal window) to facilitate light penetration to the brain, it is quite possible that the actual amount of energy reaching the brain of the eels was more or less the same as the amount that made the blinded Phoxinus react.

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## LEGENDS

Table 1 Summary of results and experimental schedule which was followed in order to attain light energy thresholds for the synchronization of locomotor activity to a given LD regime.

Fig. 1 Locomotor activity responses to different Zeitgeber regimes. Line under x-axis denotes dark period.  
y-axis = pooled hourly activity as per cent deviation from the period mean activity.

x-axis = time axis in hours.

a. LD 12:12 conditions - white light  $20 \mu\text{W}/\text{cm}^2$

b. DD conditions

c. LD 12:12 conditions - white light  $0.02 \mu\text{W}/\text{cm}^2$

d. LD 12:12 conditions - white light  $0.05 \mu\text{W}/\text{cm}^2$

e. LD 12:12 conditions - white light  $0.09 \mu\text{W}/\text{cm}^2$

f. LD 12:12 conditions - 548 nm  $0.004 \mu\text{W}/\text{cm}^2$

g. LD 12:12 conditions - 548 nm  $0.006 \mu\text{W}/\text{cm}^2$

h. LD 12:12 conditions - 665.700 nm  $0.009 \mu\text{W}/\text{cm}^2$

i. LD 12:12 conditions - 403 nm  $0.005 \mu\text{W}/\text{cm}^2$

Fig. 2 Relation between light intensity and number of eels synchronizing to applied LD regime.

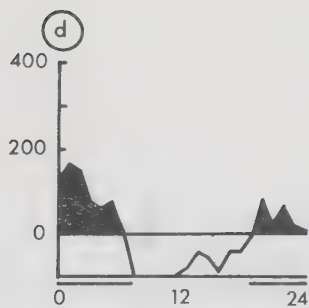
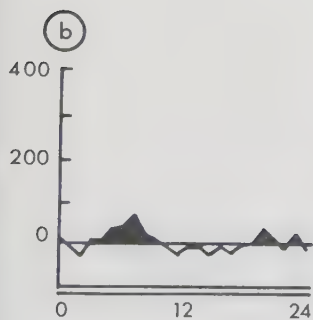
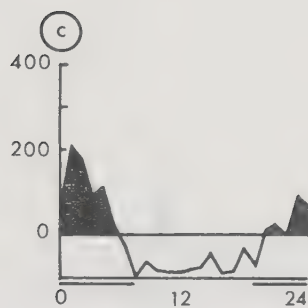
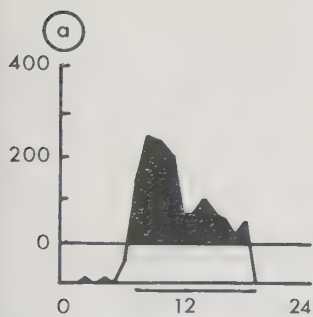
White light = filled quadrants

Light of  $\lambda$  548 nm = open quadrants

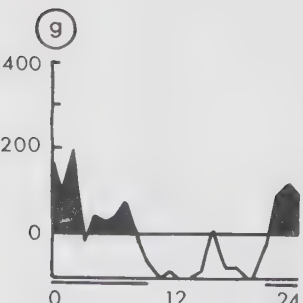
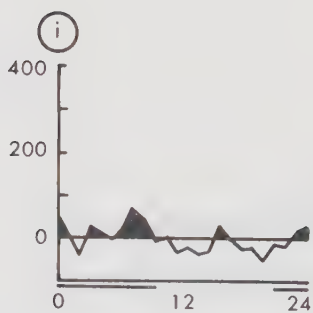
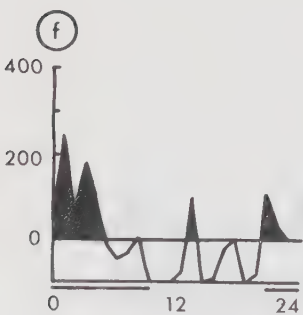
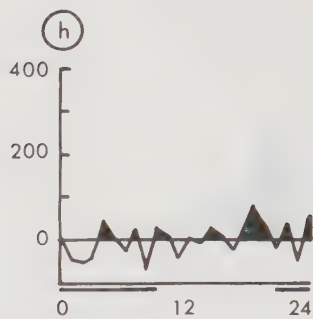
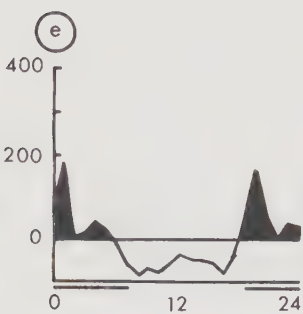
Table 1

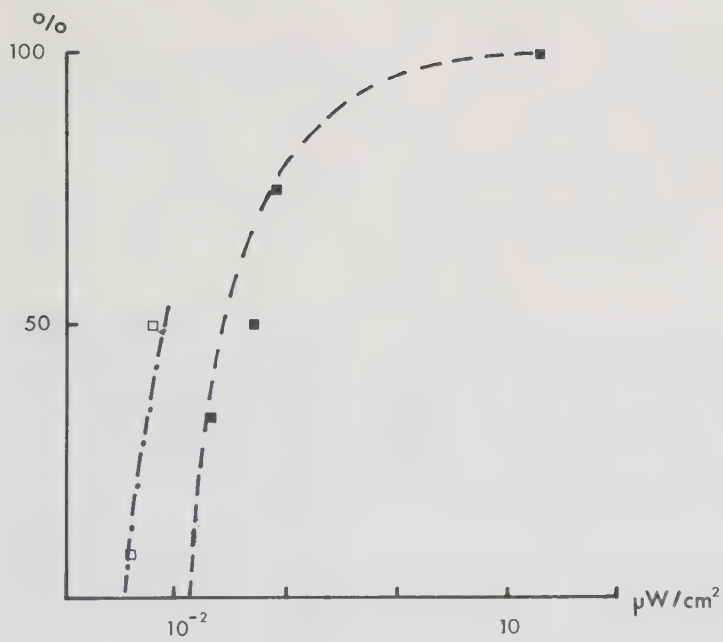
| Light/dark regime | Phase shift | Light quality                  | Light intensity                       | Number of days | Synchronized locomotor activity (no. of eels) |
|-------------------|-------------|--------------------------------|---------------------------------------|----------------|-----------------------------------------------|
| LD 12:12          |             | white 3400°K                   | 20 $\mu\text{W}/\text{cm}^2$          | 43             | 12/12                                         |
| DD                |             | dark                           | $< 10^{-7}$ $\mu\text{W}/\text{cm}^2$ | 18             | 0/12                                          |
| LD 12:12          | 180°        | white 2750°K                   | 0.02 $\mu\text{W}/\text{cm}^2$        | 11             | 4/12                                          |
| LD 12:12          |             | white 2750°K                   | 0.05 $\mu\text{W}/\text{cm}^2$        | 5              | 6/12                                          |
| LD 12:12          |             | white 2750°K                   | 0.09 $\mu\text{W}/\text{cm}^2$        | 6              | 9/12                                          |
| LD 12:12          | 30°         | 548 nm $\Delta\lambda = 16$ nm | 0.004 $\mu\text{W}/\text{cm}^2$       | 6              | 1/12                                          |
| LD 12:12          |             | 548 nm $\Delta\lambda = 16$ nm | 0.006 $\mu\text{W}/\text{cm}^2$       | 5              | 6/12                                          |
| LD 12:12          |             | 665-700 nm                     | 0.009 $\mu\text{W}/\text{cm}^2$       | 5              | 0/12                                          |
| LD 12:12          |             | 403 nm $\Delta\lambda = 16$ nm | 0.005 $\mu\text{W}/\text{cm}^2$       | 5              | 0/12                                          |





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## Spectral Characteristics of Visible Radiation Penetrating into the Brain and Stimulating Extraretinal Photoreceptors

### Transmission Recordings in Vertebrates

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**Summary.** Supravital recordings of spectral transmission in the brains of two species of teleosts (*Anguilla anguilla*, *Ictalurus nebulosus*), an amphibian (*Rana temporaria*), a reptile (*Lacerta muralis*), two species of birds (*Passer domesticus*, *Columba livia*), and a mammal (*Phodopus sungorus*) indicate that photons of longer wavelengths (700–750 nm) penetrate approximately 1,000 times more effectively into the hypothalamus than photons of shorter wavelengths (400–450 nm). The decrease in transmission from 750 to 400 nm is slightly interrupted by a plateau around 500 to 540 nm because of the transmission characteristics of hemoglobin. There is a small, ill-defined transmission minimum around 430 nm corresponding to the transmission minimum of melanin and hemoglobin (soret band). The high light sensitivity of deep diencephalic photoreceptors involved in the control of photoneuroendocrine events characteristic of some non-mammalian vertebrates suggests the occurrence of photopigment-containing receptors and nerve cells summing the input of several photoreceptors. However, in addition to photopigments, there may also exist other photosensitive compounds that mediate non-visual photoneuroendocrine responses.

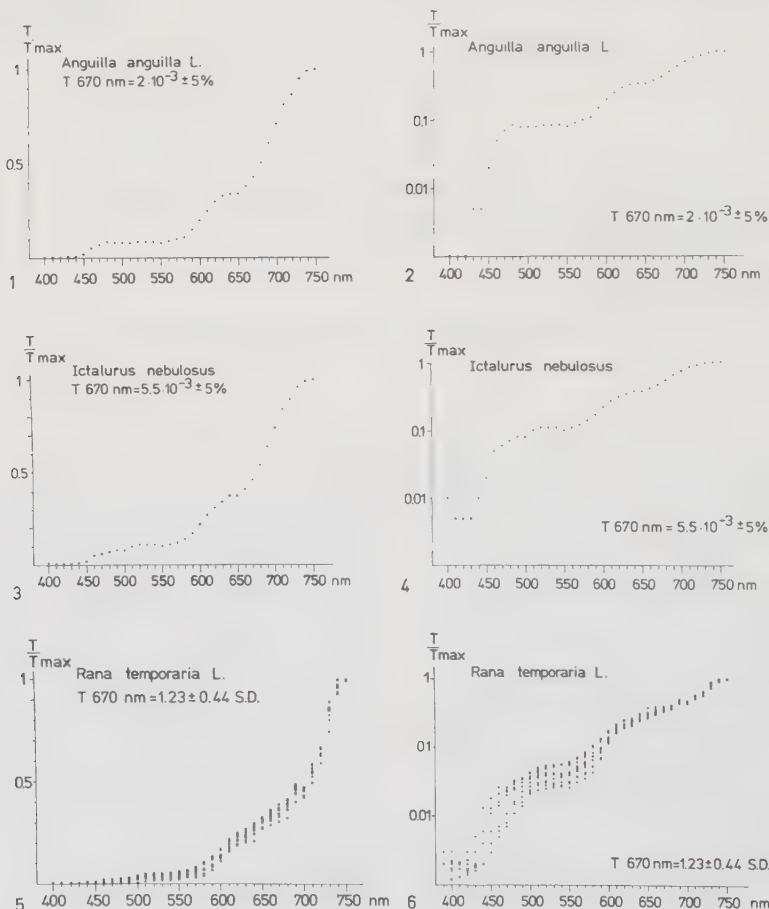
### Introduction

Vertebrates, with the apparent exception of mammals, have photosensitive areas in the diencephalon that are most probably located in the vicinity of the third ventricle. Stimulation of the “deep diencephalic photoreceptor” in blinded and pinealectomized European minnows, *Phoxinus phoxinus* (L.), results in a color change (von Frisch, 1911) or in light dependent motor reactions (light-dependent conditioned reflexes, Scharer, 1928; for the role of the pineal organ in color change mechanisms of *Phoxinus phoxinus*, see also Schäfer, 1964). Van Veen et al. (1976) showed that in blinded and pinealectomized European eels, *Anguilla anguilla* (L.), deep diencephalic photoreceptors are responsible for (1) the synchronization of circadian motor activity with an external photoperiod and (2) for the control of photonegative behavior. In birds, the photoperiodic control of gonadal growth is apparently mediated by the deep diencephalic photoreceptor and not by the rods and cones of the lateral eyes (for review, see Benoit, 1970; Menaker, 1971a, b; Farner, 1973; Yokoyama et al., 1978). There is very little information on the nature and location of the deep diencephalic photosensitive elements and their connections with neuroendocrine and motor effectors. Hartwig (1975), using microspectrophotometric techniques, detected in *Phoxinus phoxinus* a circumscribed ependymal area covering the antero-dorsal hypothalamus that contained a photosensitive compound with an ill-defined absorption maximum between 550 and 580 nm. This region is rich in nerve cells contacting the cerebrospinal fluid with bulbous cilia of the 9+0 type, thus resembling early developmental stages of retinal and pineal photoreceptor cells

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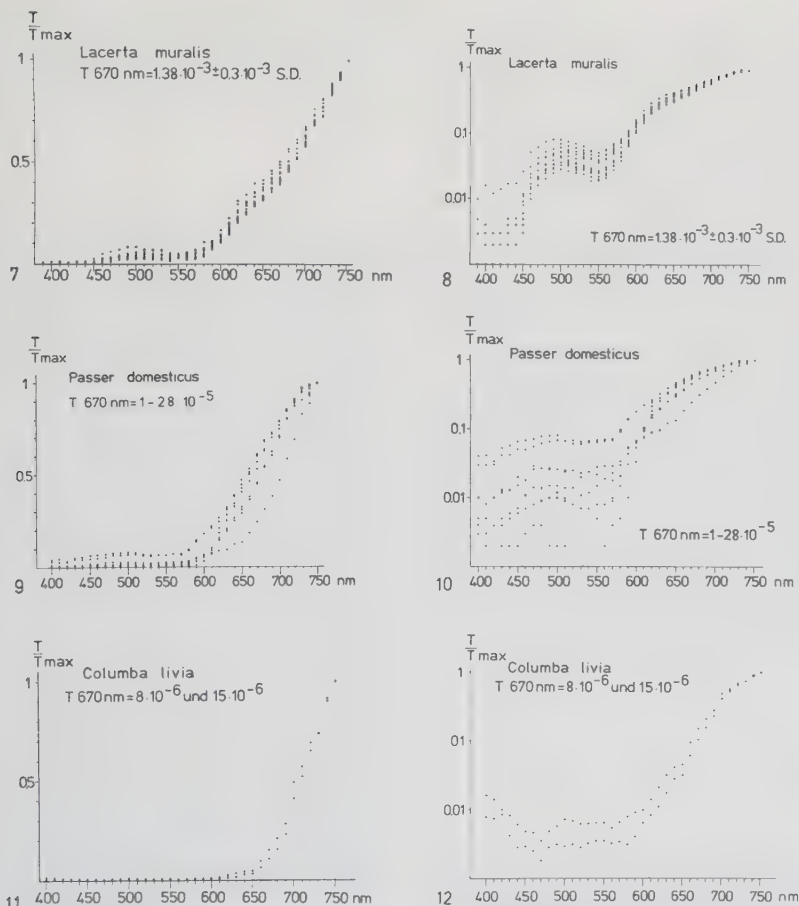
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Figs. 1-6. Spectral transmission recordings of light penetrating into the hypothalamus in teleosts (*Anguilla anguilla*, *Ictalurus nebulosus*,  $n=10$ ) and amphibians (*Rana temporaria*,  $n=10$ ) exhibiting a melanophore index of 3-4. In Figs. 1-4 each dot represents the mean of 10 individual measurements. In Figs. 5 and 6 each dot represents a single measurement. Figures show corrected instrument readings expressed as  $T$  (transmission) divided by  $T_{\max}$  (maximal transmission value). Absolute values of transmission are given at 670 nm. Transmission values are plotted in a linear (Figs. 1, 3, 5) or in a log (Figs. 2, 4, 6) scale

(Oksche and Hartwig, 1975). Light reaching the diencephalon must penetrate skin, bony skull, meningeal connective tissue, blood vessels, and brain substance. On its way the light is (1) scattered and (2) the spectral composition is modified by a color-filter effect (e.g. hemoglobin, melanin) of the penetrated

tissue. Since deep diencephalic photoreceptors that mediate photoperiodic responses analyze the amount of light reaching the brain at different phases of the internal clock (for references, see Yokoyama et al., 1978), it is worthwhile to measure (1) the amount of light that reaches the diencephalon and (2) the



Figs. 7-12. Spectral transmission recordings of light penetrating into the hypothalamus in lizards (*Lacerta muralis*,  $n=12$ ) and in birds (*Passer domesticus*,  $n=8$ ; *Columba livia*,  $n=2$ ). Each dot represents one single measurement. The figures show corrected instrument readings expressed as  $T$  (transmission) divided by  $T_{\text{max}}$  (maximal transmission value). Absolute values of transmission are given at 670 nm. Transmission values are plotted in a linear (Figs. 7, 9, 11) or in a log (Figs. 8, 10, 12) scale

spectral absorption characteristics of the tissues that cover the diencephalon.

## Materials and Methods

Spectral transmission was recorded supravitaly in the brains of male and female species of 1) teleosts (10 European eels, *Anguilla*

*anguilla*, of about 60 g body weight and 10 bullheads, *Ictalurus nebulosus*, 20 g); 2) amphibians (10 frogs, *Rana temporaria*, 23-40 g); 3) reptiles (12 lizards, *Lacerta muralis*, 3-6 g); 4) birds (8 House sparrows, *Passer domesticus*, 19-28 g and 2 white feathered pigeons, *Columba livia*, 267 and 340 g); and 5) one mammal (a male dsungarian dwarf hamster, *Phodopus sungorus*, 25 g). Animals were killed by decapitation. The dorsal aspect of the skull (skin, bones and tissues) was left intact. The ventral surface of

the brain was carefully removed of the covering tissues and subsequently the hypophysis and the caudal-most part of the infundibulum were exposed. Spectral transmission was recorded with a modified microspectrophotometer (Beckman Instruments MS 1206) equipped with a Xenon high-pressure lamp (Osram XBO 450) and interchangeable photomultiplier tubes (spectral response type S-20 and spectral response type S-13). The power supply of the Xenon arc and of the photomultiplier tube exhibited a high stability (uncontrollable changes less than 0.6%). The illumination microscope of the microspectrophotometer was modified to produce a homogeneously illuminated area of  $3 \times 3$  cm at the level of the object stage. The specimens were placed upside down on a glass slip on the homogeneously illuminated stage and the previously exposed ventral brain surfaces inspected using the observation microscope of the instrument by means of epiillumination. In a field covering an area from the caudal border of the transversal commissure (teleosts) or the optic chiasma (amphibians, reptiles, birds, mammals) to the dissected infundibulum, the penetrating light was measured in 10 nm intervals from 400 to 750 nm. The spectral band width was 10 nm. In measurements at wavelengths longer than 640 nm in addition to the manufacturer's filter for suppression of undesired harmonics of the grating monochromator, a blocking filter (Schott OG 630) was used to eliminate false light output. After an adaptation of the high tension supplying the photomultiplier tube, the measurements were performed with a stabilized high tension (500–800 V in small sized specimens and 1,200–1,400 V in large sized specimens). Uncorrected instrument readings showed values between 0.5–155 units (total scale range: 0.1–164 units). Correcting factors were obtained in the following way: by means of a variable diaphragm inserted into the illuminating system at a point of ray parallelism the intensity of the illuminated area was reduced to values recordable with the high tension selected for the recording of the specimen. Reduction of illumination intensity did not interfere with the spectral composition or with the aperture of the illuminating system. Corrected instrument readings were expressed as  $T$  (transmission) divided by  $T_{\max}$  (maximal transmission value). Finally, absolute values of transmission were obtained at 670 nm using a calibrated series of neutral density filters (Schott).

## Results

### General Aspects

In all species investigated visible radiation of longer wavelengths penetrates into the diencephalon approximately 1,000 times more effectively than photons of shorter wavelengths. The longest wavelength investigated (750 nm) shows the best penetration to the hypothalamus. The shape of the transmission curve (plotted in a linear scale of  $T$  values) in general depends on the absolute size of the skull and brain. In large specimens (mammal, bird) the decrease of transmission values is more rapid in comparison to small specimens (teleost, amphibian). The decrease of transmission values is slightly interrupted at wavelengths between 500–540 nm. Between 420 and 440 nm there exists a small, ill-defined transmission minimum. In large specimens the transmission curve

plotted in a linear scale of  $T$  values decreases rapidly from maximal values at 750 nm to values near zero around 600 to 650 nm. In transmission curves plotted in a logarithmic scale of  $T$  values the further decrease of transmission values is clearly visible (c.f. Figs. 1–12).

### Special Aspects

*Anguilla anguilla*, *Ictalurus nebulosus*, *Rana temporaria*. In the two species of teleosts and in the anuran the absolute number of photons that penetrate into the hypothalamus depends strongly on the melanophore index (MI) of the specimen investigated. In a pilot study some animals were placed for several days prior to investigation on a white or a black continuously illuminated background. The melanophore index (MI) was recorded according to Hogben and Slome (1931) on the pectoral fins (teleosts) or the interdigital web (*Rana temporaria*) by means of a stereo microscope. In animals adapted to a white background (MI=1–2) the amount of light reaching the hypothalamus is about 100 times higher than in animals adapted to a black background (MI=4–5). However, the adaptation to a white or to a black background does not interfere with the general pattern of the transmission curves. The results shown in Figs. 1–6 are obtained in animals exhibiting a MI of about 3–4 (for transmission data of tissues covering the pineal organ in *Salmo irideus*, *Rana temporaria* and *Rana esculenta*, see Morita, 1966).

*Passer domesticus*, *Columba livia*, *Phodopus sungorus*. In these larger animals it was necessary to record longer wavelengths with a photomultiplier of the spectral response type S-20 and shorter wavelengths with a photomultiplier tube of the spectral response type S-13. Moreover, in the pigeon and in the dsungarian dwarf hamster only animals bearing either white feathers or a white winter fur could be measured with the required precision. In large specimens bearing dark feathers or a dark fur only photons of wavelengths longer than 600 nm could be detected due to technical limitations of the instrument used in this investigation. However, in these specimens (recordings in three dark feathered pigeons, in two dark feathered chickens and in a dsungarian dwarf hamster bearing a dark summer fur) the decrease of transmission values followed the same principles as in small specimens.

House sparrows were trapped and investigated in January. Thus, the reported values deal with animals that are known to be maximally photosensitive. Recordings in the *Phodopus sungorus*, bearing a white



winter fur exhibit transmission values in the range of  $10^{-7}$ – $10^{-8}$  for wavelengths between 650–750 nm. Values recorded below 550 nm are characterized by a poor signal to noise ratio. Therefore, the data are not shown in a diagram.

## Discussion

It is well known that measurable quantities of visible radiant energy penetrate the skull into the hypothalamus, even in mammals (Ganong et al., 1963). In the House sparrow, *Passer domesticus*, the light intensity of a full moon night (moon not covered by clouds) is sufficient to stimulate the photoreceptor located inside the brain (for review, see Menaker, 1971a, b; for threshold data of light sensitive pineal organs, see Dodt, 1973). Menaker and coworkers (McMillan et al., 1975) demonstrated that electromagnetic radiation between 600 and 700 nm characterized by an energy of only 0.15 erg/cm<sup>2</sup>/s measured at the head surface of *Passer domesticus* is capable of penetrating feathers, skin, bony skull and brain tissue and inducing gonadal growth. In blinded ducks Benoit (for review, see Benoit, 1970) found that longer wavelengths stimulated gonadal growth more effectively than shorter wavelengths when the radiation was applied externally.

The light sensitivity of the deep diencephalic photoreceptors demonstrated in *Passer domesticus* by McMillan et al. (1975) is comparable to that of retinal and pineal photoreceptors which contain photopigment molecules. In experiments dealing with the spectral sensitivity of deep diencephalic photoreceptors, it is necessary to know the spectral transmission values of the tissues covering the diencephalon, because the number of photons reaching the diencephalon per unit time differs with the wavelengths applied. The presented transmission recordings show that in all species investigated longer wavelengths from 700 to 750 nm penetrate 100–1,000 times more effectively into the diencephalon than shorter wavelengths from 400 to 450 nm. Double-beam transmission recordings performed in isolated skin pieces of *Anguilla anguilla* and in protein microdroplets containing synthetic melanin, revealed that the black pigment melanin has a characteristic absorption spectrum with a high transmission of long wavelengths and a low transmission of short wavelengths. Moreover, a less distinct transmission minimum was observed between 420 to 440 nm (Hartwig and van Veen, unpublished). In general, the transmission spectrum of melanin resembles the spectra of this study. The small plateau between 500–540 nm found in the present recordings most probably is due to hemoglobin transmission maxima,

characteristic for this range (for general considerations concerning the influence of blood transmission characteristics on the spectral sensitivity of retinal photoreceptors see Dodt, 1958; Peregrin, 1974). Three factors seem to be responsible for the slope of the transmission spectra observed: (1) photons of long wavelengths are known to be scattered to a lesser extent by biological tissues and thus to penetrate deeper than photons of shorter wavelengths (c.f. Seliger and McElroy, 1965), (2) melanin and (3) hemoglobin (erythrocytes of the blood vessels) show a strong absorption in the visible range of the spectrum. Hemoglobin and melanin both absorb short wavelengths to a greater degree than long wavelengths. The absolute number of photons penetrating into the diencephalon per unit time is a function of the size of the animal and of the density of melanin in the tissues covering the brain.

The absolute amount of environmental light reaching deep diencephalic photoreceptors must be higher in living animals than the values reported in this investigation for the following reasons: 1) Measurements were performed at the ventral surface of the hypothalamus and not at probable sites of the diencephalic photoreceptors. 2) Deep diencephalic photoreceptors are most probably located in the vicinity of the third ventricle; thus from a dorsal direction environmental light must penetrate only the relatively thin layers covering the dorsal diencephalic roof; the cerebrospinal fluid absorbs only negligible amounts of photons in the visible range of the spectrum. 3) Environmental light penetrates the skull not only from dorsal but also from lateral, anterior and posterior directions whereas in this investigation, due to technical reasons, only the dorsal skull surface could be illuminated. However, for experiments dealing with the spectral sensitivity of diencephalic photoreceptors in intact animals the knowledge of the spectral transmission characteristics of the tissues covering the photoreceptor is more important than the knowledge of the absolute number of photons penetrating into the brain. Finally it should be noted that in aquatic animals daylight is modified by the transmission and reflectance characteristics of water.

In vertebrates lamellated photoreceptor cells have been found only in the retina and in the pineal organ (Teleostei, Amphibia, Lacertilia). Retina and epiphysis cerebri have developed phylogenetically as diencephalic evaginations. Most probably a circumscribed region of the diencephalic primordium is the only area in vertebrates capable of forming photoreceptor cells. This assumption is strongly supported by a report of Sacerdote (1971) who showed differentiation of ectopic retinal structures in the hypothalamo-hypophyseal area of *Triturus crist-*

tatus induced by a methylene blue-soaked barrier at the level of the median eminence. Photoreceptor cells containing regularly lamellated outer segments only exist in connection with specialized supporting cellular elements (e.g. pigment epithelium in the retina; c.f. Hollyfield and Witkovsky, 1974). Regularly lamellated outer segments are responsible for a high optical density of photopigments in a circumscribed structural area.

This condition is mandatory in an image-analyzing photoreceptor system. The deep diencephalic photoreceptor does not serve to analyze spatial intensity differences. It records the number of photons reaching the diencephalon. This task can be fulfilled by unlamellated outer segments distributed over a large space, whereas in the retina one photon reaching one single outer segment must be detected in order to attain optimal spatial analysis at low intensities.

In considering the exact structure, function and location of deep diencephalic photoreceptors, one cannot preclude the possibility that additional mechanisms of photosensitivity may be of importance. Such possibilities include (1) photosensitive enzymes (Hug et al., 1971), (2) oscillatory behavior of enzymic activity induced by radiant energy (Comorosan et al., 1969), and (3) enzyme activation or inhibition by photolabile chromophores (e.g. carotenoids, hemoproteins; Deal et al., 1969). However, as far as is known the latter mechanisms would have a decided disadvantage in that their photosensitivity is much lower than that of photopigments.

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EXTRARETINAL AND EXTRAPINEAL PHOTORECEPTION IN THE  
EEL, Anguilla anguilla

Synchronization of locomotor activity to visible  
radiation of different wavelengths and intensities

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## SUMMARY

The synchronization of locomotor activity to threshold intensities of the Zeitgeber-regime was investigated in blinded and pinealectomized European yellow eels. The different wavelengths applied (447-700 nm) were corrected to an equal number of quanta reaching the diencephalon. Synchronization by extraretinal and extrapineal photoreceptors took place when the photoperiod consisted of wavelengths of 447 nm, 497 nm, 548 nm ( $\Delta \lambda = 16$  nm). Long wavelengths ( $\lambda$  665-700 nm) failed to stimulate the extraretinal and extrapineal photoreceptors at a comparable energy level. This suggests a broad maximum of the sensitivity range of the encephalic photoreceptor in the European yellow eel, adapted to the diversified light milieu of its environment.

## INTRODUCTION

Since von Frisch (1911) reported extraretinal and extra-pineal photoreception in the European minnow, and Scharrer (1928) located the site of this photoreception to the diencephalon, a number of non-mammalian species have been shown to possess encephalic photoreception (for reviews, see: Underwood 1979, Wolken and Mogus 1979).

By using microspectrophotometric methods Oksche and Hartwig (1975) demonstrated a photolabile compound in the wall of the third ventricle in the European minnow (see also Hartwig 1975). In the same area they found bulbous cilia of sensory type (9x2+0) protruding into the cerebrospinal fluid. Similar bulbous cilia are found in developmental stages of retinal and pineal photoreceptors and in the pineal stalk of adult European minnows (for a review, see: Oksche and Hartwig 1979).

Pineal photoreceptors of functional type are present in the European eel (Oksche 1965, R  deberg 1971), as in other fish (for reviews, see: D  dt 1973, Hamasaki and Eder 1977, Vollrath 1981).

Besides the pineal organ, the eel possesses a parapineal organ (R  deberg 1971). R  deberg (1969) found photoreceptor cells with fully developed outer segments in the parapineal

organ of the rainbow trout and anti-opsin positive cells were demonstrated in the parapineal organ of the Chinese silver carp, the carp, the goldfish, and the pike perch by Vigh-Teichmann, Röhlich, Vigh, and Aros (1980). However, the parapineal organ of at least young eels (elvers) does not contain any photoreceptor cells with fully developed outer segments (van Veen 1981).

The involvement of extraretinal and extrapineal photoreception in the synchronization of locomotor activity has been demonstrated in the European eel (van Veen, Hartwig, and Müller 1976) and in the lake chub (Kavaliers 1980).

The locomotor activity pattern of the European yellow eel is nocturnal (Müller 1972, van Veen et al. 1976, Westin and Nyman 1979). The threshold intensity of visible radiation ( $\lambda$  548 nm  $\Delta\lambda$  = 16 nm) in the light phase of an LD regime, synchronizing the locomotor activity of intact eels, was determined to  $0.006 \mu\text{W}/\text{cm}^2$  by van Veen and Andersson (1981).

The aim of the present investigation was to study extraretinal and extrapineal photoreception entraining locomotor activity cycles at threshold intensities in the European yellow eel in order to reveal spectral sensitivity characteristics of the extraretinal and extrapineal photoreceptor.

## MATERIAL AND METHODS

Eight small yellow eels (Anguilla anguilla L.) (body-weight 2-3 g), supplied from an eel culture plant, were used in the present study. The animals were kept single in tanks containing 2 l of aerated running tap water constant in temperature ( $23.5^{\circ}\text{C}$  with randomly distributed changes of  $0.5^{\circ}$ ). All tanks were provided with a sandy bottom and hiding places (clam shells and/or small stones).

In these tanks, in which the locomotor activity of the animals was to be recorded, the animals were kept for more than 5 weeks prior to the investigation, in order to adapt them to the laboratory conditions. The animals were fed a surplus of Tubifex at the commencement of the experiments. As the Tubifex thrived in the sandy bottom food was constantly available.

Seven days prior to the start of the experiments the eels were bulb- and pinealectomized. After the surgical treatment they were kept in constant conditions (DD).

### Surgical technique

The animals were anaesthetized in a solution of Urethane in water (35 g/l) until the gill movements stopped. During the surgical treatment the animals were wrapped in wet paper towels to prevent desiccation.



Ophthalmectomy: Following a median cut through the skin of the dorsal head, the dorsal skull bones and the orbital cavities were exposed by dissecting the covering muscles. Subsequently, the external eye muscles and the optic nerves could be transected and the eye bulbs removed.

Pinealectomy: Subsequently, after dissecting the covering muscles a piece of bone overlying the pineal region was cut out by using microscissors. The saccus dorsalis containing the pineal vesicle and part of the pineal stalk was then visible through the meninx. The meninx was opened and the saccus dorsalis together with the pineal vesicle, the pineal stalk, and parapineal organ were removed. The opened meninx was replaced and the skin sutured with silk.

The locomotor activity of the eels was recorded by a computerized laboratory equipment provided with infrared photocell units (van Veen, Andersson and Nilsson 1981). The applied LD regimes included dusk and dawn periods of 0.5 h. Underwater light intensities were recorded with a radiometer. Intensities of light with narrow bandwidth were changed by a phase-controlled lamp dimmer and a diaphragm at the focal point of the lamp. Light quality was modified by double band interference filters ( $\Delta\lambda$  16 nm;  $\lambda = 448, 497, 548$ ), or a long-pass cut-off filter ( $\lambda = 665$  nm) and a short-pass cut-on filter ( $\lambda = 700$  nm) (van Veen et al. 1981).

As different wavelengths of visible radiation penetrate to various degrees into the brain of vertebrates (Hartwig and van Veen 1979), care was taken to correct the energy content of the different wavelengths applied to an equal number of quanta reaching the diencephalon (Fig. 1).

Table 1 shows the experimental schedule which was followed, in order to attain the spectral sensitivity characteristics of the extraretinal and extrapineal photoreceptor, synchronizing locomotor activity to a given LD regime. The individual animal's ability to respond to an LD regime was tested by an artificial photoperiod of strong white light (3400°K) at the end of the experiment. The colour temperature of the light was measured with a PROFISIX-photometer (Gossen) provided with a PROFI-color (Gossen) accessory device.

In order to evaluate the performed surgical incisions, the eels were decapitated at the end of the experiments. The brains were dissected out, fixed in Carnoy's fluid, embedded in paraffin, serially sectioned at 6  $\mu$ m, and stained with Ehrlich's haematoxylin and eosin. Then the sections were studied in the light microscope.

## RESULTS

The examination of the sectioned brains revealed no remains of pineal tissue and except for one animal the parapineal organs had also been completely removed. No signs of regeneration of pineal or parapineal tissue were found.

As mentioned above, a light regime with an energy content of white light far above threshold values ( $20 \text{ uW/cm}^2$ ,  $3400^\circ\text{K}$ ) was applied to test the individual animal's ability to respond to an artificial photoperiod. Two of the eight animals did not pass this test and were consequently considered as unable to respond to an LD regime. Thus, in the evaluation of the results these two animals were excluded and the following outcomes are based on the remaining six eels.

Different light regimes were analysed in the following sequences:

LD 12:12 conditions -  $\lambda 548 \text{ nm } 0.009 \text{ } \mu\text{W/cm}^2$  (approx.  $2 \times 10^{10}$  quanta/s  $\text{cm}^2$ ).

When subjected to these conditions, four of the six eels synchronized their locomotor activity to the Zeitgeber regime (Fig. 2a).

Constant conditions - DD less than  $10^{-7} \mu\text{W}/\text{cm}^2$

Offered constant conditions, the animals displayed an apparently arrhythmic behaviour pattern (Fig. 2b).

LD 12:12 conditions -  $\lambda$  448 nm  $0.05 \mu\text{W}/\text{cm}^2$  (approx.  $1 \times 10^{11}$  quanta/s  $\text{cm}^2$ ) - phase shift of  $90^\circ$  compared to the LD regime prior to DD conditions.

Four out of the six eels synchronized the locomotor activity to this Zeitgeber regime (Fig. 2c).

LD 12:12 conditions - 665-700 nm  $0.001 \mu\text{W}/\text{cm}^2$  (approx.  $3 \times 10^9$  quanta/s  $\text{cm}^2$  calculated for  $\lambda$  680 nm) - phase shift of  $90^\circ$  compared to previous LD regime.

None of the animals showed synchronized locomotor activity under this LD regime.

LD 12:12 conditions -  $\lambda$  497 nm  $0.009 \mu\text{W}/\text{cm}^2$  (approx.  $2 \times 10^{10}$  quanta/s  $\text{cm}^2$ ) - phase shift of  $90^\circ$  compared to previous LD regime. Three of the six eels synchronized the locomotor activity to this Zeitgeber regime.

Table 1 shows the summarized results.

## DISCUSSION

This study showed that four out of six young eels synchronized their locomotor activities to a photoperiod containing radiation of  $\lambda 548 \text{ nm}$  ( $\Delta\lambda = 16 \text{ nm}$ ) with  $2 \times 10^{10} \text{ quanta/s cm}^2$  in the light phase. van Veen and Andersson (1981) found that 6 out of 12 intact eels showed synchronized locomotor activity when subjected to a similar LD regime ( $\lambda 548 \text{ nm}$  and  $1.6 \times 10^{10} \text{ quanta/s cm}^2$ ). Considering the number of eels synchronizing in these two investigations one may conclude that the threshold for synchronization is approximately the same for intact eels as for blinded and pinealectomized ones. The number of quanta reaching the retinal- respectively extraretinal photoreceptors is, however, different due to absorption characteristics of the overlying tissues (Hartwig and van Veen 1979). It is worth mentioning that the dark adapted visual apparatus of the closely related Anguilla rostrata (Gordon, Shapley, and Kaplan 1978) exhibits a maximal sensitivity of at least three log units below the threshold values synchronizing locomotor activity rhythms at  $\lambda 548 \text{ nm}$ .

In a previously performed study on extraretinal and extrapineal photoreception in the eel (van Veen et al. 1976), it was reported that the eyes are involved in the synchronization process. Reanalysing those earlier results

under view of the recent data dealing with threshold intensities obtained in the present investigation, it must be questioned if the experimental conditions of the earlier investigations were optimal for demonstrating the involvement of the eyes. The covering experiments included intact and bulbectomized eels. In the intact eels, the lateral parts of the skull and orbital cavities could not be covered to the same extent as in the bulbectomized animals. Because of the high intensity of white light used in the light phase, probably considerable amounts of light penetrated aside of the cover to stimulate the extraretinal photoreceptive system. Thus, it is reasonable to reconsider the role of the eyes in the process of synchronization in the eel and postulate that they are not involved in the process. Studies on intact animals provided with an opaque cover will be performed at threshold intensities of the Zeitgeber, in order to determine the role of the lateral eyes in the synchronization process (cf. investigations by Menaker and Underwood 1976 in the house sparrow).

Photoperiodic regulation of gonadal growth in birds and lizards has been shown to depend on extraretinal photoreception (for reviews , see: Underwood 1979, 1980). The encephalic photoreceptive area involved is located to the hypophysial tuberal area (Yokoyama, Oksche, Darden, and Farner 1978). These intercranial systems are most

sensitive to extracranially applied longer wavelengths 665-700 nm (cf. Menaker and Underwood 1976). In this investigation care was taken to correct the energy content of the different wavelengths in order to provide the encephalic photoreceptor with a similar number of quanta per area and time at each selected wavelength. Under these conditions the extraretinal photoreceptor did not show its maximal sensitivity in that area of visible radiation. Instead the medium and short wavelengths were stimulative. The provided light regime with radiation of  $\lambda 548$  nm or  $\lambda 447$  nm resulted in the largest number of synchronized animals. No eels showed synchronized behaviour to radiation with a spectral composition of 665-700 nm. This suggests a fairly broad spectral sensitivity range of the encephalic photoreceptor in the medium wavelengths. Because water is an effective barrier for long and short wavelengths (working as a monochromator with a broad bandwidth, Lythgoe 1972), an extraretinal receptor functioning in the long and short wavelengths of the visible radiation would be of little use for the eel. Kavaliers (1980) tested in the lake chub extraretinal sensitivity to longer wavelengths. That the blinded and pinealectomized lake chub can synchronize locomotor activity to these wavelengths is possibly an adaptation to its habitat. This is also reflected in a retinal sensitivity of the long wavelengths.

The retinal photoreceptors of the yellow eel contain photopigments with a maximal sensitivity at 501 and 523 nm, and lack photopigments sensitive to the long wavelengths of the visible spectrum (Gordon et al. 1978). Photopigments sensitive to long wavelengths are demonstrated in the lake chub (Kavaliers 1980). This adaptation to environmental lighting conditions (Loew and Lythgoe 1978) is probably also reflected in the sensitivity maxima of the extraretinal and extrapineal photoreceptive system(s). Any differences of transmission characteristics between the here studied small yellow eels, and that of the bigger eels used by Hartwig and van Veen (1979), ought to be limited to the absolute amount of penetrating visible radiation. However, some differences could also exist in the spectral absorption due to lower concentrations of absorbing active substances (hemoglobin, melanin etc.). This could influence the here demonstrated absorption maximum by narrowing its sensitivity range.

In teleosts, no definite location or structural basis of the extraretinal and extrapineal photoreceptors has been demonstrated, although lesion experiments in Phoxinus phoxinus (Scharrer 1928) indicate that the receptor is located in the vicinity of the third ventricle.



As mentioned above (see: Introduction) a photolabile compound and structures resembling developmental stages of retinal and pineal photoreceptors have been found in the wall of the third ventricle. In the same area in the eel, Hartwig and van Veen (unpublished findings) observed a photolabile compound, but this showed an extremely low optical density. Furthermore, results of microspectrophotometric transmission recordings in relatively thick sections of tissue are difficult to evaluate because they can be distorted by scattering phenomena within the analysed tissue.

The histological examination of the pinealectomized specimens revealed that the parapineal organ of one of the eels was intact. Since photoreception could be the function of this organ (van Veen, in preparation), at least in some teleost fish, an intact parapineal organ may be involved in the synchronization process. However, no difference in the synchronization of locomotor activity to the provided Zeitgeber regimes could be detected in the eel with the intact parapineal organ compared to those in which the parapineal organ was extirpated. Therefore, it is suggested that this organ is not to a major extent involved in the perception of the Zeitgeber.

Kavaliers (1979) showed that in teleost fish kept under constant conditions, the locomotor activity is affected

if the animals are pinealectomized. This phenomenon has also been demonstrated in passerine birds, although some discrepancies are apparent in the reported results (cf. Gwinner 1978).

When a desynchronization of the locomotor activity was displayed by the blinded and pinealectomized eels, it occurred rather soon after the Zeitgeber regime was abolished. This could be due to a rapid disintegration of loosely coupled oscillators (see Eriksson and van Veen 1980). The resynchronization process was also rather fast (one to two days). These quick responses to a phase shift of the Zeitgeber have also been observed in the house sparrow and in lizards (Menaker and Underwood 1976; Underwood and Menaker 1976). Naturally, it is questionable if such a quick response to a Zeitgeber regime implicates actual entrainment and synchronization (for detailed definitions of the terms "entrainment" and "synchronization" see Aschoff 1965). Much is dependent on the internal state of the biological oscillator, its frequency and range of entrainment. Animals with a synchronized endogenous (free running) rhythm, which differs only slightly from the natural oscillator will show a long entrainment and synchronization process. However, if the endogenous rhythm runs free with a period well different from 24 hours (e.g. 20 h), the entrainment process will only take a few days. If the observed locomotor activity coincides with

the changes of the Zeitgeber without reflecting the endogenous rhythm, a masking phenomenon has occurred. It is known that the eel possesses a phototactic behaviour (van Veen et al. 1976; Edel 1979). This behaviour can mask a biological rhythm. As threshold levels of the Zeitgeber were used in the present study, the phototactic stimulus was probably not very strong. In any case, in order to display a masking, the driving oscillator, which the photoperiod implicates, has to be perceived.

These results suggest a broad sensitivity range of the encephalic photoreceptor in the European yellow eel reflecting an adaptation to the diversified environmental light milieu.

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## LEGENDS

Table 1. Summary of results and experimental schedule which was followed in order to attain spectral sensitivity characteristics of the extraretinal and extrapineal photoreceptor.

Fig. 1. No. of quanta/s  $\text{cm}^2$  applied at studied wavelengths (solid line) and transmission of light penetrating into the hypothalamus of the eel at these wavelengths (transmission values from Hartwig and van Veen 1979). T = transmission,  $T_{\text{max}}$  = maximal transmission value. Values are plotted in log scales.

Fig. 2. Examples of locomotor activity responses of blinded and pinealectomized eels to different Zeitgeber regimes.

Line under x-axis denotes dark period.

y-axis = pooled hourly activity as per cent deviation from the period mean activity.

x-axis = time axis in hours.

a = LD 12:12 conditions  $\sim 548 \text{ nm } 0.009 \mu\text{W}/\text{cm}^2$

b = DD conditions

c = LD 12:12 conditions  $\sim 448 \text{ nm } 0.05 \mu\text{W}/\text{cm}^2$

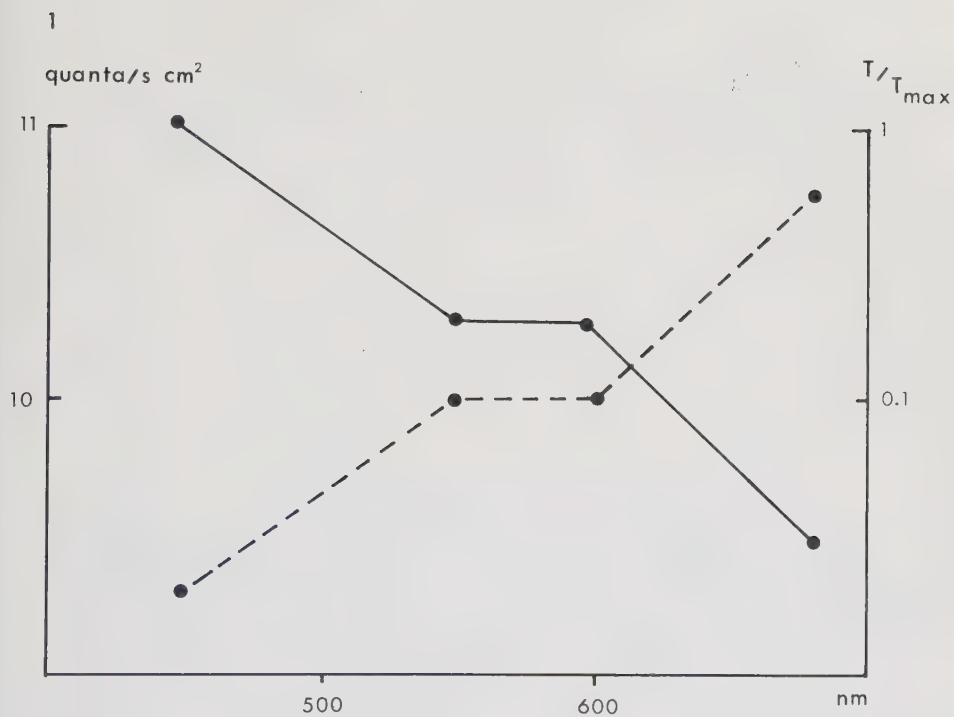
d = LD 12:12 conditions  $\sim 665\text{--}700 \text{ nm } 0.001 \mu\text{W}/\text{cm}^2$

e = LD 12:12 conditions  $\sim 497 \text{ nm } 0.009 \mu\text{W}/\text{cm}^2$

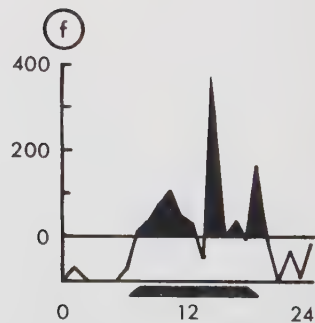
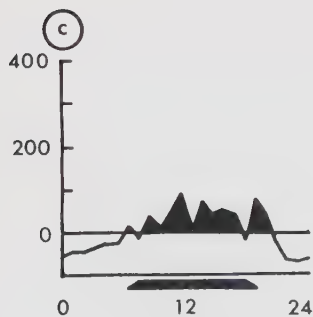
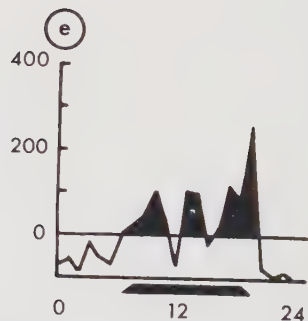
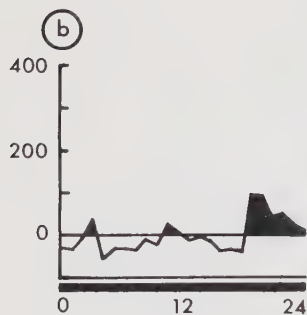
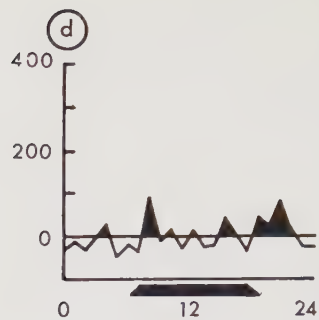
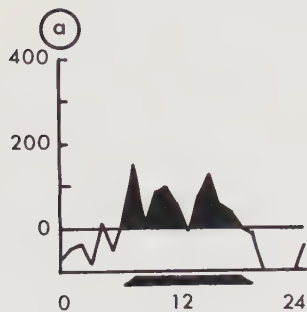
f = LD 12:12 conditions  $\sim \text{white light } 20 \mu\text{W}/\text{cm}^2$

Table 1

| Light/dark regime | Phase shift | Light quality                                           | Light intensity                          | Number of days | Synchronized locomotor activity (no. of eels) |
|-------------------|-------------|---------------------------------------------------------|------------------------------------------|----------------|-----------------------------------------------|
| DD                |             | dark                                                    | $< 0.1 \text{ } \mu\text{W}/\text{cm}^2$ | 7              | -                                             |
| LD 12:12          |             | $\lambda 548 \text{ nm } \Delta\lambda = 16 \text{ nm}$ | $0.009 \text{ } \mu\text{W}/\text{cm}^2$ | 18             | 4                                             |
| DD                |             | dark                                                    | $< 0.1 \text{ } \mu\text{W}/\text{cm}^2$ | 4              | -                                             |
| LD 12:12          | $90^\circ$  | $\lambda 448 \text{ nm } \Delta\lambda = 16 \text{ nm}$ | $0.05 \text{ } \mu\text{W}/\text{cm}^2$  | 12             | 4                                             |
| LD 12:12          | $90^\circ$  | 665-700 nm                                              | $0.001 \text{ } \mu\text{W}/\text{cm}^2$ | 10             | 0                                             |
| LD 12:12          | $90^\circ$  | $\lambda 497 \text{ nm } \Delta\lambda = 16 \text{ nm}$ | $0.009 \text{ } \mu\text{W}/\text{cm}^2$ | 10             | 3                                             |
| LD 12:12          |             | white light (3400°K)                                    | $20 \text{ } \mu\text{W}/\text{cm}^2$    | 6              | 6                                             |



2



THE PINEAL AND PARAPINEAL ORGANS OF THE ELVER  
(Anguilla anguilla L.): A Light- and Electron  
Microscopic Study

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## SUMMARY

The parapineal organ of the glass eel (elver) consists of c. 400 cells and is situated to the left of the entrance of the pineal stalk to the third ventricle. A prominent nerve tract containing c. 350 fibers arises from the parapineal organ and runs in connection with the habenular commissure towards the left habenular nucleus. The dominating cell type of the parapineal organ is a neuron of small diameter provided with atypical cilia (9x2+0, or rarely 8x2+0 types). Well-developed photoreceptor outer segments are lacking, and no interstitial cells of ependymal type have been observed with certainty in the parapineal organ of the elver. The axonal processes from the nerve cells form the tract leaving the parapineal organ.

The pineal organ proper consists of photoreceptor cells with well-developed outer segments, interstitial cells of ependymal type and ganglion cells. Axons from the latter form the pineal tract, which leaves the pineal organ and runs in close contact with the subcommissural organ towards the posterior commissure. The proximal part of the pineal stalk contains only a few photoreceptor cells whose outer segments are less developed than those of the pineal body and the distal part of the stalk.

## INTRODUCTION

The pineal complex of the eel consists of a pineal organ proper and a parapineal organ (Rüdeberg, 1971).

The pineal organ proper of eels with body weights from 150 to 450 g contains photoreceptor cells with well-developed outer segments, interstitial cells of ependymal type, and ganglion cells (Rüdeberg, 1971), which accords with other investigated fishes (for review, see Falcon, 1979; Vollrath, 1981).

The parapineal organ of teleosts is considered to be a homologue of the parapineal organ of lampreys and the parietal eye of lizards (Studnička, 1905; Oksche, 1965; Rüdeberg, 1969).

In the parapineal organ of lampreys photoreceptors with fully-developed outer segments are present (Meiniel, 1971), indicating that these photoreceptors are functional units in light perception. Also, the parietal eye of the lizard contains photoreceptor cells (for review, see Quay, 1979), which have been shown to be functional (for review, see Dodt, 1973). In teleosts, the parapineal organ has long been regarded as to be present only in the young animals. Not until 1969 was the parapineal organ described in adult fish by Rüdeberg. Since then a number of adult teleosts



have been shown to possess a parapineal organ; Salmo, Esox, Anguilla (Rüdeberg, 1969, 1971), Helicolenus (Ueck and Kobayashi, 1979), Gasterosteus (van Veen et al., 1980), Carassius, Cyprinus, Hypophthalmichthys, Lucioperca (Vigh-Teichmann et al., 1980) and Phoxinus (van Veen and Borg, unpublished results).

With the electron microscopic technique, Rüdeberg (1969) demonstrated three cell types in the parapineal organ of the rainbow trout: 1) a nerve cell type, which was dominant; 2) interstitial cells of ependymal type; and 3) very few photoreceptor cells, which had fully-developed outer segments. He also stated that the parapineal organ of the rainbow trout cannot be regarded as a miniature pineal organ.

Using anti-opsin, Vigh-Teichmann et al. (1980) showed that the parapineal organs of the goldfish, carp, Chinese silver carp, and pike perch contained very few anti-opsin-positive cells situated around a labyrinth lumen.

The fact that intact, blinded, and blinded and pineal-ectomized eels exhibit light-dependent locomotor activity (van Veen et al., 1976) makes it important to study the parapineal organ of the eel as an alternative site of photoreception. In addition, the deep parts of the pineal organ and their contacts with the subcommissural organ and the third ventricle are investigated as a possible site of photoreception.

## MATERIAL AND METHODS

Twenty elvers (glass eels), Anguilla anguilla L. of stage 6 (Bertin, 1956) were obtained in the river Viskan in western Sweden. The specimens were used immediately or kept in captivity for c. 30 days in tanks with low water temperature (8°C) and short-day LD 8:16 conditions.

### Light microscopy

Entire heads of ten specimens were fixed in Bouin-Hollande (Romeis, 1968) or 80% ethanol-formaldehyde acetic acid (18:1:1), embedded in paraffin and serially sectioned at 7-8  $\mu$ m. They were then stained with Azan or Holmes' silver method as modified by Blest (1961) using 2,6-dimethylpyridine.

The number of cells present in the parapineal organ was estimated by counting cell nuclei on camera lucida drawings (objective x100 with 1,25 aperture) of Holmes and Azan stained serial sections (3 specimens). Peripherally cut parts of nuclei and peripheral glial elements were not counted.

### Electron microscopy

After decapitation of ten specimens the brain was rapidly dissected, the skull being opened to facilitate the

penetration of the cold fixative during the dissection procedure. The following fixatives were used: 1) 5% glutaraldehyde in 0.05 M Na-cacodylate buffer (pH 7.4) for 2-4 h. (4 animals); 2) 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) for 2-4 h. (4 animals); and 3) 2%  $\text{OsO}_4$  in 0.01 M Na-cacodylate buffer (pH 7.4) for 2-4 h. (2 animals).

After postfixation in cacodylate buffered 2-4%  $\text{OsO}_4$  for two hours, the specimens were block-stained in 0.5% phosphotungstic acid dissolved in 100% ethanol for 30 min and then embedded in Vestopal W.

Silver-to-grey ultrathin sections were prepared from the entire parapineal organ and part of the pineal organ in frontal, horizontal and sagittal planes. Most of the sections were post-stained in 1% uranyl acetate with 75% ethanol and 0.5% lead citrate in distilled water. They were then examined with a JEOL 100 STEM instrument.

The number of axons in the tract leaving the parapineal organ were counted on electron micrographs of a transversely sectioned tract at the point of its emergence from the parapineal organ.

## RESULTS

### Light microscopy

#### The parapineal organ

The parapineal organ of the elver is situated to the left of the entrance of the pineal stalk into the third ventricle at the subcommissural organ (SCO) (Figs. 2, 3). In silverimpregnated material a connection with the left habenular nucleus by a prominent nerve tract (Fig. 3) is observed. This tract runs rostrally in contact with the habenular commissure prior to entering the left habenular nucleus.

The parapineal organ of these small eels is about 100  $\mu\text{m}$  in diameter, oval in shape and consisting of c. 400 cells. The organ is surrounded by a connective tissue capsule having an opening exclusively for the nervous tract. No connections other than that with the left habenula are found.

The cells of the parapineal organ are homogeneously distributed within the parenchyma and exhibit uniform diameters (c. 10  $\mu\text{m}$ ). They all have the same appearance with a round nucleus occupying the major part of the perikaryon and the cytoplasm staining very weakly when stained with Azan. Centrally in the organ there seems to be a lumen. No

photoreceptor outer segments are noted to project into this lumen, as seen in the pineal organ proper of the elver.

#### The pineal organ

The pineal organ of the elver consists of a corpus and a stalk (Fig. 1), the lumen of which is rather narrow and opens into the third ventricle at the SCO (Fig. 3).

Photoreceptor cells are present, as are interstitial cells. The pineal tract, which consists of axonal protrusions from the ganglion cells in the pineal body and stalk, intermingles with fibers of the posterior commissure.

#### Electron microscopy

##### The parapineal organ

The structure of the parapineal organ is shown in Fig. 4. The dominating cell type of the parapineal organ carries one or two cilia originating from a pit in the cell (Figs. 8, 9, 12). The axonem of the cilia consists of  $9 \times 2 + 0$  tubuli (Fig. 9). Occasionally one double tubulus is missing, and the  $8 \times 2 + 0$  configuration is thus displayed (Fig. 10). Each cilium carries a centriole basally (Fig. 12). No kinocilia could be detected.

Band desmosome-like structures are present between the cell bodies in the middle part of the organ (Fig. 11). In the perikarya a few vesicles containing electron-dense material surrounded by a unit membrane are observed. No synaptic vesicles or other synaptic specializations could be detected. Myelin bodies are present within some cells in one of the preparations. Rough endoplasmic reticulum is mostly observed in the apical parts of the cell body. The rounded nucleoplasm exhibits a weak and homogeneous electron density (Figs. 5, 6). A poorly developed Golgi apparatus (Fig. 7) as well as a relatively low number of mitochondria are present in the apical cellparts. Axonal processes project from the cell bodies and form a nerve tract leaving the parapineal organ and running towards the left habenular nucleus (Figs. 5, 6). Neurotubuli ( $\varnothing \approx 40\text{--}50$  nm), endoplasmic reticulum and mitochondria are present in these axon-like processes. In two specimens, the nerve bundle leaving the parapineal organ consists of c. 350 fibers.

In the periphery of the parapineal organ parenchyma, flattened cellular elements resembling glial cells are found in contact with the basal lamina. Other cells with nuclei situated more centrally in the parapineal organ resemble astrocytes possessing long slender protrusions which make contact with the basal lamina. The cytoplasm of these elements stains more intensively than that of other cell types. No macrophages were observed.

## The pineal organ

The pineal organ of the elver consists of photoreceptor cells with fully-developed outer segments, comparable to those of the fully grown eel (Rüdeberg, 1971). Synaptic ribbons are frequently observed in photosensory elements of the pineal body and the stalk (Figs. 14, 15). In addition, interstitial cells of ependymal type and ganglion cells are present. The slender stalk of the pineal organ makes contact with the subcommissural organ (SCO) and the lumen of the pineal organ opens into the third ventricle at the SCO. The most proximal interstitial cells of the pineal stalk contact the specialized ependymal elements of the SCO without showing membrane specializations (Fig. 13).

The interstitial cells of ependymal type predominate in the pineal stalk ventral to the habenular commissure. However, a few photoreceptors can also be present. The outer segments of these photoreceptor cells are not as well developed as those higher up in the stalk and in the pineal body.

From the posterior wall of the pineal stalk, the pineal tract runs in close contact with the SCO (Fig. 13) toward the posterior commissure.

No fiber connections could be detected between the pineal tract and the habenular commissure nor with the parapineal organ. Some macrophages are observed in the pineal stalk and body but none are seen in the parapineal organ.

## DISCUSSION

The present investigation has demonstrated that the parapineal organ of the elver contains one dominating cell type carrying cilia. These cilia are only moderately transformed, lacking the central tubuli. From the cell bodies axons project toward the left habenular nucleus, forming a nerve tract.

Cells carrying atypical cilia situated within the central nervous system of vertebrates are regarded as possessing a sensory function (for review, see Leonhardt, 1980). The foremost examples of this are the primary sensory cells of the photosensory and olfactory systems. In the first case, however, the cilia are greatly transformed during development. Early developmental stages of retinal and pineal photoreceptors do not show the major modifications of their ciliar membrane, i.e., fully developed regularly lamellar invaginations of the cilia membrane (for review, see Oksche and Hartwig, 1979). The ultrastructure of these elements resembles that of cerebrospinal fluid (CSF) contacting neurons of the paraventricular organ and preoptic



nucleus of teleosts that are also regarded to be sensory (Vigh and Vigh-Teichmann, 1974; Vigh et al., 1975). The preoptic nucleus of the eel exhibits, in addition to CSF-contacting neurons, small neurons which carry atypical cilia (9x2+0) projecting into the intercellular space (Vigh-Teichmann et al., 1976).

On the basis of the morphological characteristics of the majority of its cells, i.e., small neurons carrying atypical cilia, the parapineal organ of the elver can be regarded as sensory.

If proposing a chemoreceptive function of the parapineal organ, it must be noted that the organ is surrounded by a capsule of connective tissue; no open connection with the CSF is present. Moreover, in the center of the organ where most atypical cilia are found, desmosome-like cell contacts are frequently present. Tight junctions limiting extracellular diffusion of substances are, however, not observed. In addition, blood vessels are in close contact with the connective tissue capsule. Likewise, as in the previously mentioned small neurons of the preoptic nucleus (Vigh-Teichmann et al., 1976) the cilia of the parapineal organ project into the intercellular space. The atypical cilia of the parapineal organ without major specializations, do not rule out chemoreception. The sensory cells in the parapineal organ of the elver, however, do not in all

respects correspond to the sensory cells of the olfactory epithelium of the eel (Schulte, 1972).

Nevertheless, a possible photosensory function of the parapineal organ must also be considered, since in other investigated teleosts photoreceptive elements are demonstrated within the parapineal organ. This is also the situation in the parietal eye of the lizard (for review, see Quay, 1979) and the parapineal organ of the lamprey (Meiniel, 1971). These structures are regarded as homologues with the parapineal organ of teleosts (Studnička, 1905; Friedrich-Freksa, 1932; Oksche, 1965). The sparse amount of photoreceptors demonstrated in the parapineal organ of the rainbow trout and the existence of anti-opsin positive cells demonstrated in the parapineal organs of some other teleosts, could be a reflection of the site of origin of this organ, i.e., the primordium of the epithalamic diencephalon, which possesses the capacity to develop photoreceptor elements.

The lack of sensory cells with fully developed outer segments in the parapineal organ of the elver, and the comparatively few photoreceptor cells found in the same organ of other teleosts, suggest another sensory function if the photoreceptive is disproved.

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## LEGENDS

### PLATE I

Light micrographs of the pineal complex of the elver.

Scale markers 25  $\mu$ m.

Fig. 1. Sagittal section through the habenular commissure showing the pineal organ.

Fig. 2. Horizontal section through the habenular commissure, the pineal stalk and the parapineal organ.

Fig. 3. Transverse section through the subcommissural organ (star) showing the opening of the pineal stalk lumen (arrowhead) into the third ventricle, and the parapineal tract (arrow).

Abbreviations: ch, commissura habenularis; l, pineal lumen; nh, nucleus habenularis; p, pineal organ; pa, parapineal organ; ps, pineal stalk; sd, saccus dorsalis; to, tectum opticum; vt, velum transversum.

Fig. 4. Schematic representation of the parapineal organ of the elver. Ca, capsule; Ci, cilium; D, desmosome-like junction; G, glial-type cell; PAT, parapineal tract; S, sensory cell.



## PLATE II

Electron micrographs of the parapineal organ of the elver.

Fig. 5. Transverse section through the parapineal organ (pa) and the parapineal tract (pat). Note the densely packed, small, parapineal neurons. Scale marker 5  $\mu\text{m}$ .

Fig. 6. Axonal processes (arrowheads) from the parapineal small neurons in the middle of the organ. Scale marker 1  $\mu\text{m}$ .

## PLATE III

Electron micrographs of the parapineal organ of the elver.

Fig. 7. An active Golgi complex in the parapineal organ. Scale marker 0.5  $\mu\text{m}$ .

Fig. 8. A cilium (arrow) extending from a small parapineal neuron towards the middle of the parapineal organ. Scale marker 0.5  $\mu\text{m}$ .

Fig. 9. A cilium (arrow) and two ciliary bases (arrowheads) in the same neuron in the parapineal organ. Scale marker 0.5  $\mu\text{m}$ .

Fig. 10. An 8x2+0 cilium (arrowhead) in the parapineal organ. Scale marker 0.2  $\mu$ m.

Fig. 11. Desmosome-like structure between the apical parts of two small neurons. Scale marker 0.1  $\mu$ m.

#### PLATE IV

Fig. 12. Electron micrograph of the parapineal organ of the elver. Small parapineal neuron with cilium (arrowhead) and centriole (arrow). Note the basal process (bp) of the adjacent parapineal neuron. nu, nucleus of parapineal neuron. Scale marker 1  $\mu$ m.

#### PLATE V

Electron micrographs of the pineal stalk of the elver.

Fig. 13. The entrance of the pineal stalk close to the subcommissural organ. Note the ependymal cells (e) of the pineal stalk, the neural elements (n) in close proximity to the subcommissural organ and the endoplasmic cisternae (stars) in the cells of the subcommissural organ. Scale marker 1  $\mu$ m.

Fig. 14. Synaptic ribbons (arrowhead) in a photoreceptor cell near the lumen of the pineal stalk. Scale marker 1  $\mu\text{m}$ .

Fig. 15. Synaptic ribbons (arrowhead) in the basal elongation of a photoreceptor cell close to the basal lamina (bl) of the pineal stalk. Scale marker 1  $\mu\text{m}$ .

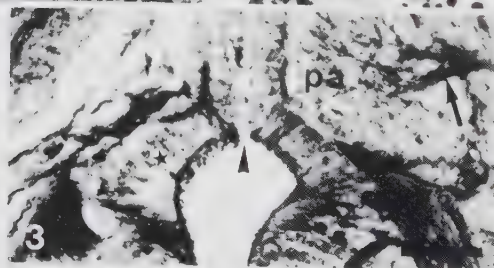
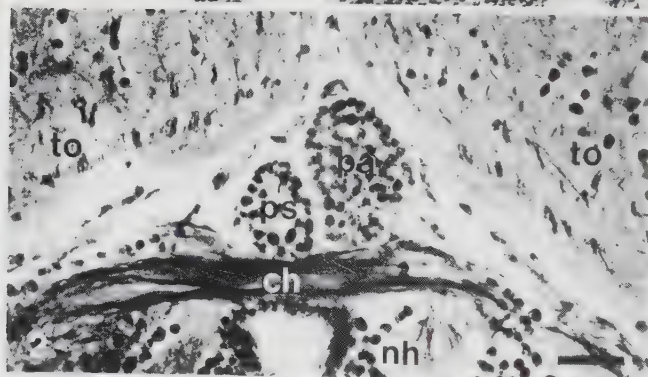
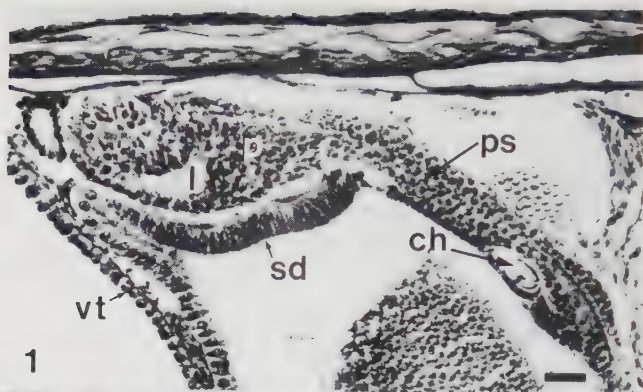
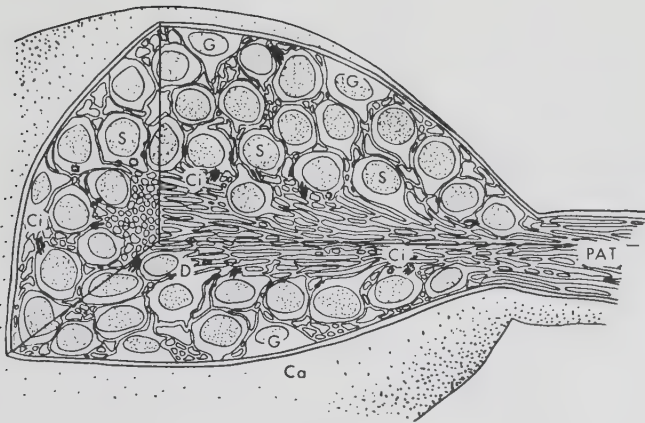
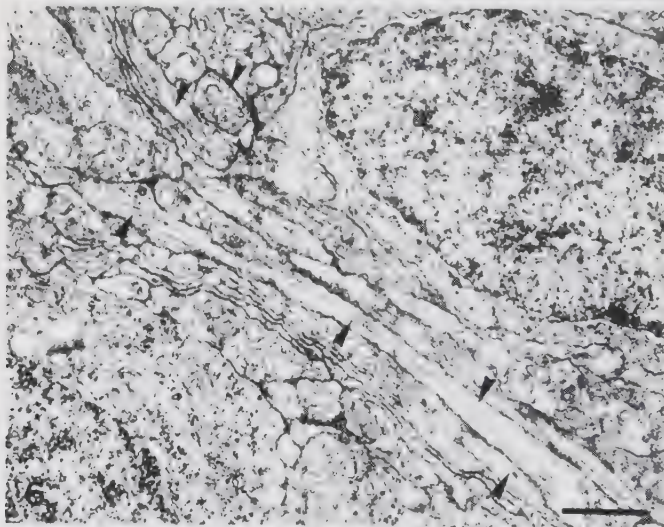
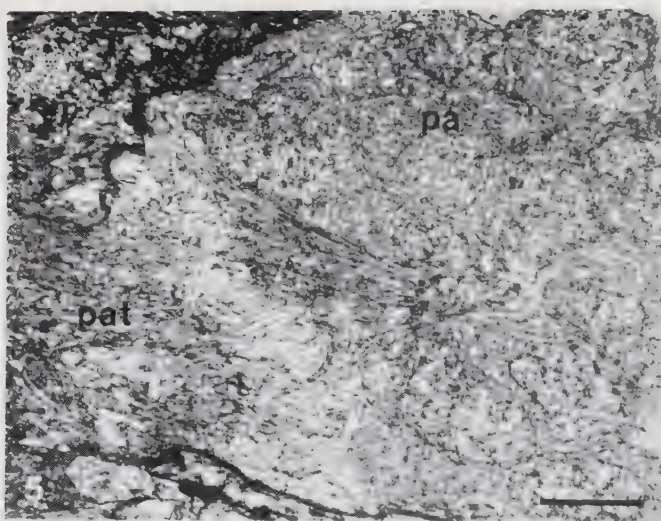
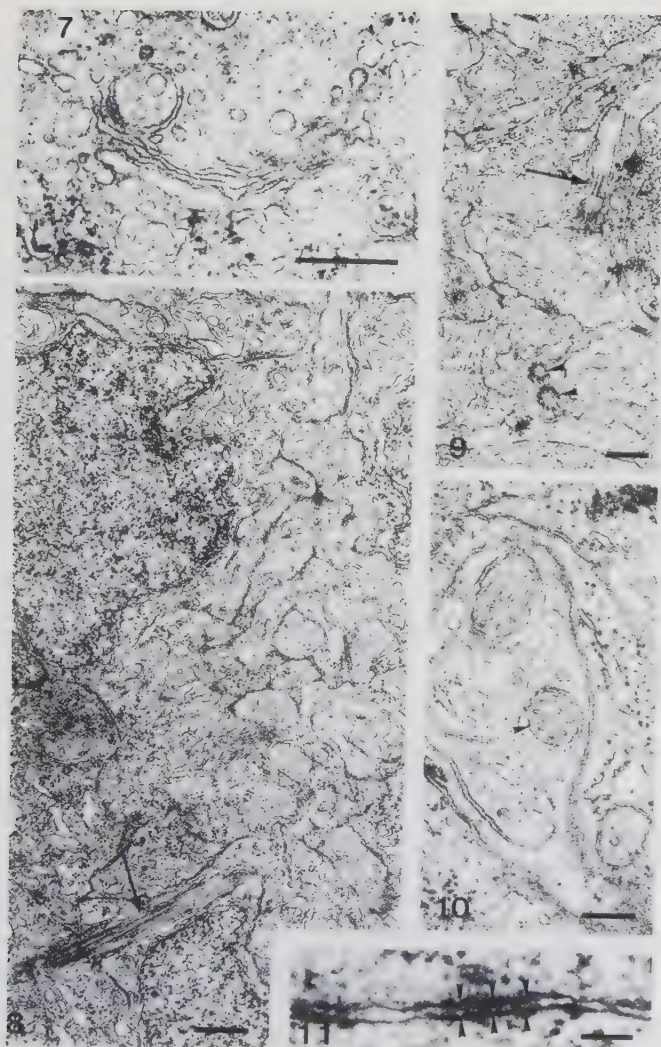


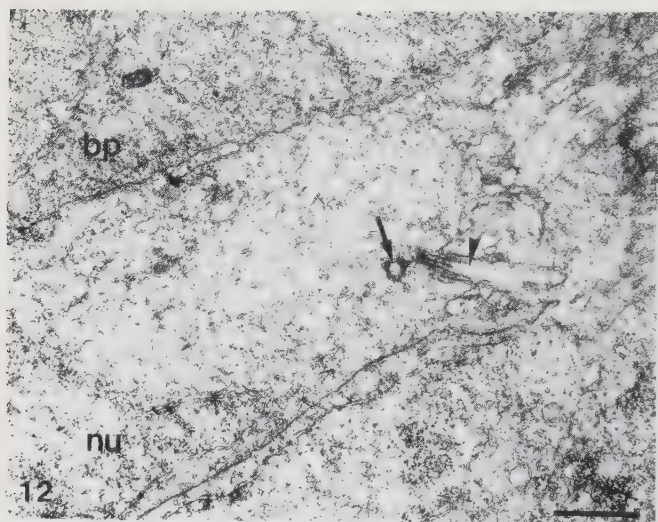
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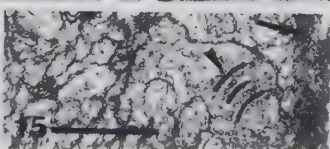
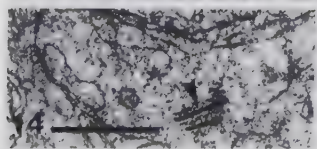
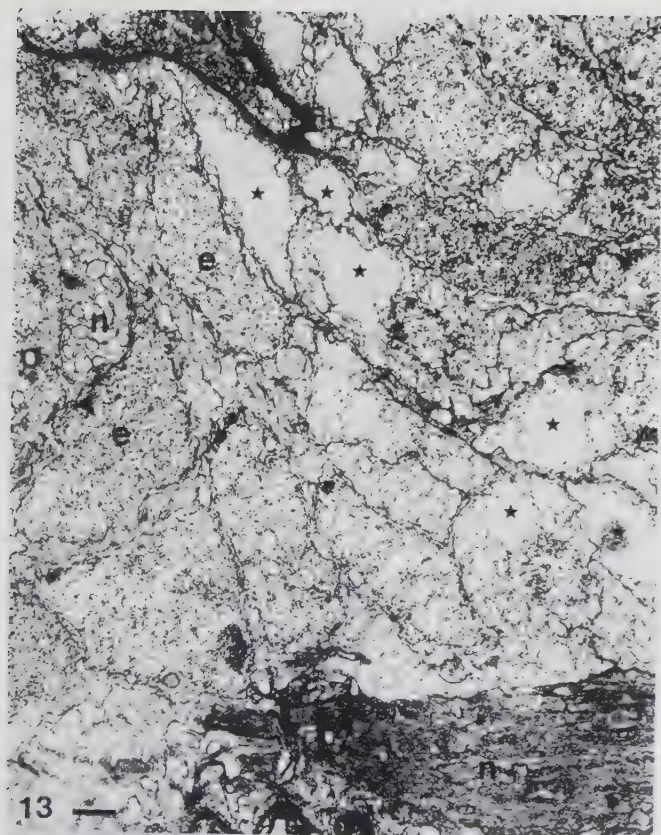














DIURNAL VARIATION OF 5-HT CONTENT IN THE PINEAL  
ORGAN OF THE YELLOW EEL (Anguilla anguilla L.)

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Summary. For determination of the amount of 5-hydroxy-tryptamine (5-HT) in the pineal organ and serum of the yellow eel, single or pooled pineal organs and blood were sampled and investigated using high-performance liquid chromatography with electrochemical detection. The eels were kept under a strict light regime of LD 12:12 for 5 weeks prior to commencement of the investigation. The peak level of 5-HT content in the pineal organ was found at the dark-to-light transition, whereas the lowest content was recorded in the middle of the light phase and at the light-to-dark transition. A relatively high 5-HT content was also present in the middle of the dark phase. The serum content of 5-HT, however, showed no such pronounced diel fluctuation.

## INTRODUCTION

The indoleamine 5-hydroxytryptamine (5-HT, serotonin) is present in the pineal organ of many vertebrates. In many amniotes the 5-HT content of the pineal organ has been found to manifest considerable circadian fluctuation. In the most extensively studied species, the rat, the peak content is in the middle of the light period and the lowest amount in the middle of the dark period (Quay, 1963). Peaks in pineal 5-HT content have been found in the middle of the dark period in Gallus (Meyer et al., 1973), near the dark-to-light transition in Mustela (Yates and Herbert, 1979), Columba (Quay, 1966a), Coturnix (Hedlund et al., 1971) and Lacerta (Petit and Vivien-Roels, 1977) and near the light-to-dark transition in Macaca (Quay, 1966b), Lacerta (Petit and Vivien-Roels, 1977) and Testudo (Vivien-Roels et al., 1979). In the penguin, Pygoscelis, pineal serotonin content was higher in the middle of the night than in the middle of the day (De Gallardo and Piezzi, 1973).

Using the formaldehyde-induced fluorescence method, 5-HT and/or its precursor 5-hydroxytryptophan (5-HTP) have been demonstrated in the pineal organ of many vertebrates (for review, see Møller and van Veen, 1980) including some teleosts: Atherinopsis, Salmo (Hafeez and Quay, 1969), Esox (Owman and R  deberg, 1970; Falcon et al., 1980), and Gasterosteus (van Veen et al., 1980). Meissl et al. (1978) demonstrated 5-HT in the pineal organ of Salmo

employing  $^{14}\text{C}$  dansylation combined with microchromatography, but they failed to demonstrate any significant difference in 5-HT content in light and darkness.

A circadian 5-HT rhythm has been found in whole brains of Fundulus (Fingerman, 1976) and in the hypothalamus of Carassius (Delahunty et al., 1978; Olcese et al., 1980). However, to our knowledge, no diurnal 5-HT rhythm has been demonstrated in the pineal organ of any anamniote to date; neither has the absolute amount of 5-HT been determined in anamniotic species.

The 5-HT content of mammalian blood is mainly found in the platelets (Weissbach and Redfield, 1961). Circadian variations in blood 5-HT have not been widely studied, but diurnal fluctuations have been found in whole blood in chickens (Meyer et al., 1973) and in serum + platelets in rats (Scheving et al., 1972). These fluctuations are not as marked as those found in the pineal organ.

In the present study, the content of the 5-HT in the pineal organ and blood of the European eel, Anguilla anguilla L. at different periods of the day was determined using high-performance liquid chromatography with electrochemical detection.

## MATERIAL AND METHODS

### Untreated Individual Eels

Five yellow eels were decapitated in the middle of the light period of the day (natural photoperiod, summer). The pineal organs were rapidly dissected out and assayed individually.

### Pargyline-treated Eels

Ten yellow eels were injected intraperitoneally with the monoamine oxidase inhibitor pargyline (100 mg/kg) in order to increase the 5-HT content of the pineal organ. The eels were decapitated 2 h after injection in the middle of the light phase of the day (natural photoperiod, summer), and the pineal organs were rapidly dissected out and pooled for assay.

### Diurnal Variation

Our material consisted of yellow eels caught in The Sound (Öresund). The eels differed nominally in size, the average weight in the different groups being 47-51 g. The eels were kept in a tank with running tap water under a square light regime of LD 12:12 (lights on at 6.00). The temperature was  $\underline{c.} 14^{\circ}\text{C}$ . Before the investigation, the eels were kept under these conditions for 5 weeks, during which time they were fed ad libitum with live earthworms.

At four periods of the day (5.30-7.00; 11.30-13.00; 17.30-19.00; 23.30-01.00) c. 50 eels in each group were taken for investigation. The eels were decapitated without prior anesthesia and the pineal organs were rapidly (c. 1 1/2 min) dissected out under a dissecting microscope. In the dark periods the eels were decapitated in weak, deep-red illumination (695 nm). Deep-red light is invisible to eels (Carlisle and Denton, 1959). All pineal organs taken in each period were pooled. At the same time, 4-5 ml blood was sampled (4-5 eels) and centrifuged at 1500 rpm in order to obtain 2 ml serum. Due to the sampling methods, i.e., squeezing blood from the eels and the use of glass equipment, it can be reasonably assumed that the 5-HT of the platelets was fully released.

#### Measurement of 5-HT

The 5-HT content in the pineal organ and blood serum of eels was measured using high-performance liquid chromatography (HPLC) with electrochemical detection, maximum sensitivity being 25 pg (Hansson and Rosengren, 1978). 5-HT was extracted from the pooled pineal organ of each group with 2 ml 0.4 M perchloric acid. The pineal samples were homogenized and centrifuged at 17,000 rpm. The purification was carried out on a weakly acidic, cation-exchange column, Amberlite XE-64 (Hansson and Rosengren, 1978). The chromatographic separation was performed



on a microparticulate reverse-phase octadecyl column (5  $\mu$ m Nucleasil G<sub>8</sub>) with an aqueous solvent with an organic modifier added as the mobile phase. In all samples, an internal standard ( $\alpha$ -methyl-5-hydroxytryptamine ( $\alpha$ -Me-5-HT) was added before processing in order to estimate losses of 5-HT. The 5-HT standard was obtained from Regis chemical 430320, bought as 5-HT creatinine sulfate monohydrate, L-1828, and the internal standard  $\alpha$ -Me-5-HT from Hoffman & Roche, 3-0946/000.

## RESULTS

To determine the amount of 5-HT in the pineal organ of the yellow eel, single pineal organs were assayed. The equipment used, however, was not sensitive enough to record the low amount of 5-HT present in a single pineal organ dissected and processed under the light phase of the day. Pooling the pineal organs of 10 yellow eels that had previously been treated with monoamine oxidase inhibitor resulted in measurable quantities of 5-HT in the sample, and this was calculated to be 35 pg/pineal organ.

In order to study the diel variation of 5-HT in the pineal organ of untreated animals, 50 pineal organs were pooled and assayed four times daily. The peak level of 5-HT

content was found at the dark-to-light transition and was calculated to be 10 pg/pineal organ. The other measuring times gave the following results: middle of the light phase - below measurement sensitivity; light-to-dark transition - 1 pg/pineal organ; and the middle of the dark phase, 7 pg/pineal organ (Fig. 1). It should be mentioned that the pineal organ of yellow eels is rather small, the pineal corpus being approximately 175  $\mu\text{m}$  in diameter and 375  $\mu\text{m}$  in length.

The 5-HT content of the "serum" showed small circadian fluctuations. A peak content of 110 pg/ml "serum" was found at the dark-to-light transition. The content was 50 pg/ml in the middle of the light and dark phases and 70 pg/ml at the light-to-dark transition. Due to the limited number of samples it cannot be said if there is a circadian fluctuation in "serum" 5-HT at all.

#### DISCUSSION

The pineal organ of the yellow eel exhibits a peak in 5-HT content at the dark-to-light transition. The 5-HT content was also high in the middle of the dark period, while it was low at the light-to-dark transition and in the middle of the light period. The pattern is similar to that found in the hen (Meyer et al., 1973) and the ferret (Yates and Herbert, 1979), but very dissimilar to many other species, including the rat.

Meissl et al. (1978) found a higher content of 5-HT in the pineal organ of Salmo 6 h before lights-out than 2 h after lights-out (LD 12:12), but the difference was not significant. It is not likely that much difference in 5-HT content in the eel pineal organ would have been found if sampled on these occasions (Fig. 1).

The studies performed on pineal 5-HT rhythms are still far too dispersed for general conclusions. In this context, it is also interesting that, apart from the circadian rhythm, a circannual rhythm in the 5-HT content of the pineal organ has also been found in Lacerta (Petit and Vivien-Roels, 1977) and Testudo (Vivien-Roels et al., 1979). In the ferret the pineal 5-HT rhythm is considerably different under short-day than long-day conditions (Yates and Herbert, 1979). Furthermore, it should be kept in mind that the content of 5-HT represents the accumulated difference between uptake, synthesis, metabolism and release, and does not necessarily reflect any one of these parameters.

The small amount of 5-HT found in "serum" and the absence of a marked diurnal fluctuation makes it unlikely that the circadian fluctuation in pineal 5-HT is a secondary phenomenon to a serum 5-HT rhythm.

5-HT is further metabolized to melatonin in two steps. The activity of hydroxy-indole-0-methyltransferse (HIOMT), which catalyzes the final step, is higher during the night than during the day in the pineal organ of Salmo (Smith and Weber, 1974). The circadian rhythms and biochemistry of the pineal organ in homeothermic vertebrates have been reviewed by Deguchi (1979).

The highest 5-HT content found in untreated eels was 0.01 ng/pineal organ. This is very low compared to peak contents found in other vertebrates. e.g., c. 50 ng/pineal organ in Lacerta (Petit and Vivien-Roels, 1977), c. 50 ng/pineal organ in Testudo (Vivien-Roels et al., 1971), 90 ng/pineal organ in Gallus (Meyer et al., 1973), 1.5 ng/pineal organ in Coturnix (Hedlund et al., 1971), 280 ng/pineal organ in Columba (Quay, 1966a) and 90 ng/pineal organ in Rattus (Quay, 1963). Although there is a considerable difference in body size and size of the pineal organ in the different species, it is obvious that the 5-HT content of the pineal organ of the eel is very low. A low 5-HT content of teleost pineal organs is also shown by its weak indoleamine fluorescence in investigations with formaldehyde-induced monoamine fluorescence (Møller and van Veen, 1980).

Change in photoperiod has, in many cases, been found to act as a Zeitgeber for pineal 5-HT rhythms (e.g., Hedlund

et al., 1971; Quay, 1963, 1966a, 1966/1967; Snyder et al., 1965, 1967). The 5-HT rhythm in the pineal organ of the eel is also most likely under the influence of the light regime, although other environmental cues cannot be excluded. Light has been shown to act as a Zeitgeber for locomotor activity in the eel (e.g., van Veen et al., 1976).

The pineal organ of different amniotes receives a sympathetic innervation (for reviews see Ueck, 1979; Møller and van Veen, 1980), while the pineal organ of teleosts has never been found to receive a sympathetic (or any other) innervation (for review see Ueck, 1979). In mammals, the photoperiodic information is perceived by the lateral eyes and is conducted via sympathetic innervation to the pineal organ, where it controls indoleamine rhythms (for review see Deguchi, 1979). In the adult rat the 5-HT rhythm of the pineal organ is completely abolished after sympathectomy (e.g., Fiske, 1964; Snyder et al., 1965). Also after sympathectomy, a rhythm in pineal 5-HT is present in the ferret (Yates and Herbert, 1979) and juvenile rats (e.g., Machado et al., 1969). In the rat a considerable proportion of the 5-HT in the pineal organ is present in the catecholaminergic nerves (Bertler et al., 1964).

In contrast to mammals, birds and many reptiles, the pineal organ of teleosts contains functional photoreceptor cells

(Hamasaki and Eder, 1977). Pineal photoreceptor cells have also been found in Anguilla anguilla (Oksche and Vaupel-von Harnack, 1965; R  deberg, 1971; van Veen, 1981). This makes it likely that the photoperiodic information affecting the pineal 5-HT content is perceived by the pineal organ itself in the eel.

In many cases (e.g., Snyder et al., 1965; Machado et al., 1969; Petit and Vivien-Roels, 1977) an endogenous rhythm in 5-HT content of the pineal organ has been found. This might also be present in the eel.

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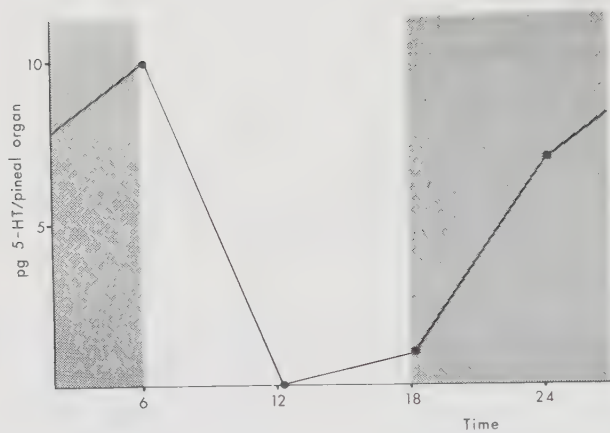


Fig. 1. Diurnal variation of 5-HT content in the pineal organ of the yellow eel. Dark fields represent dark phase of light regime (LD 12:12).





## Formaldehyde-Induced Fluorescence in the Telencephalon and Diencephalon of the Eel (*Anguilla anguilla* L.)

A Fluorescence-Microscopic and Microspectrofluorometric Investigation  
with Special Reference to the Innervation of the Pituitary

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**Summary.** In the telencephalon and diencephalon of the eel (*Anguilla anguilla* L.) formaldehyde-induced fluorescence was studied microscopically and microfluorometrically with special emphasis on the innervation of the pituitary. In the telencephalon fluorescent fibers contained predominantly noradrenaline fluorophores. Fluorescent nuclei could not be established. In the diencephalon fluorescent perikarya were found in: (1) the paraventricular organ (PVO), possessing either dopamine or, to a lesser extent, serotonin fluorophores; (2) the PVO-accompanying group, exhibiting spectral data resembling those of noradrenaline fluorophores; (3) the *nucleus hypothalami anterior* (NHA), a small paired group of catecholamine-containing cells posterior to the *commissura transversa*. — The *nucleus lobi inferioris* exhibited a high density of delicate, most probably dopamine-containing terminals, while fibers surrounding this nucleus contained noradrenaline fluorophores. A high density of fluorescent terminals containing dopamine and/or noradrenaline was also found in the habenular complex. Fluorescent terminals in the pituitary contained fluorophores resembling either dopamine or noradrenaline. Fluorescent tracts entered the pituitary from different directions. A rostral, unpaired tract enters the neurointermediate lobe, as also verified experimentally. The rostral *pars distalis* receives two paired tracts, one from a rostral and one from

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a dorsal direction. The proximal *pars distalis* also receives two paired tracts, one from a dorsal and one from a posterior direction.

**Key words:** Hypothalamus – Pituitary – Formaldehyde-induced fluorescence – Microspectrofluorometry – *Anguilla anguilla* L.

## Introduction

In the eel, *Anguilla anguilla* L., an aminergic innervation of the neurointermediate lobe of the pituitary has been described by Fremberg und Meurling (1975) using the formaldehyde-induced fluorescence technique (Falck-Hillarp technique). Aminergic fibers were also present in those branches of the neural lobe which penetrate the *pars distalis*. However, the pituitary cells are in no part of the gland directly innervated, but are separated from the neural lobe processes by an intervascular space (Knowles and Vollrath, 1966). It was suggested that the aminergic fibers are involved in the inhibitory control mechanism for MSH release (Fremberg and Meurling, 1975; Fremberg and Oliveureau, 1973).

Somewhat contradictory findings have resulted by application of the technique of Falck-Hillarp to the teleost pituitary. However, positive results, in addition to *Anguilla*, have been obtained in Conger (Iturriza, 1967), Gillichthys (Zambrano, 1970), and *Leuciscus* (Ekengren, 1975). There is no general consensus concerning the origin of the aminergic fibers in the pituitary. Knowles and Vollrath (1966), Zambrano (1970), and Urano (1971) have suggested that the *nucleus lateralis tuberis* is of aminergic nature and a source for aminergic pituitary innervation, but this has not been verified by fluorescence microscopy (Ekengren, 1975).

In teleosts investigated so far (*Salmo gairdneri*, Bertler et al., 1963; *Carrassius auratus*, Baumgarten and Braak, 1967; *Anguilla anguilla*, Lefranc et al., 1969, 1970; L'Hermite and Lefranc, 1972; *Leucoparion petersi*, Honma and Honma, 1970; *Leuciscus rutilus*, Ekengren, 1975), two aminergic nuclei have been described in the diencephalon, the *nucleus recessus lateralis* (NRL) and the *nucleus recessus posterioris* (NRP). These two nuclei most likely correspond (1) to the *organon vasculosum hypothalami* (OVH) of Scyliorhinus (Wilson and Dodd, 1973), and (2) to the paraventricular organ (PVO) and the *nucleus infundibularis dorsalis* (NID) of the Anura; (see Vigh, 1971; Terlou and Ploemacher, 1973; Prasada Rao and Hartwig, 1974).

In *Anguilla* (Lefranc et al., 1969, 1970) and *Leuciscus* (Ekengren, 1975) a nucleus has been described in the floor of the preoptic recess. It was termed *nucleus suprachiasmaticus*<sup>1</sup> in *Anguilla* and *nucleus recessus praeoptici* in *Leuciscus*. In the present paper the term *nucleus recessus praeoptici* will be retained. In the eel, Lefranc et al. (1969) in addition have described fluorescent nuclei in the posterior part of the telencephalon and in the geniculate nucleus.

The main objective of the present investigation was to study the aminergic innervation of the pituitary and to trace its possible origin. Attention was paid to fluorescent nuclei and tracts as well as to the microfluorometric identification of different monoamines in the telencephalon and diencephalon.

<sup>1</sup> This nucleus is not a homologue of the *nucleus suprachiasmaticus* of mammals



## Material and Methods

150 yellow eels (*Anguilla anguilla* L.) with an average weight of about 60 g, caught in brackish water in the Öresund near Malmö, were used. They were kept under natural lighting conditions in running tap water (14–16°C) in a tank with a dark bottom and sides. The eels were thus adapted to a black background.

Two types of investigations were performed:

### 1. Background Adaptation and Drug Treatment

- a) Thirty seven animals were placed on a white background for 3–4 h and were then killed by decapitation. The mentioned time interval was chosen because of the general increase in the specific fluorescence in the neurointermediate lobe under these conditions (Fremberg and Meurling, 1975). Immediately after decapitation the melanophore index (MI) was examined microscopically on the pectoral fins (cf. Hogben and Slome, 1931) and the brain and pituitary were freeze-dried (see below).
- b) Twenty seven animals were injected intraperitoneally with varying doses of nialamid 100–500 mg/kg body weight. These animals were placed on a white background for 3–4 h before decapitation and further treatment.

### 2. Lesion Experiments

In 70 eels the following lesion technique was employed: The animals were anesthetized by being placed in a solution of urethane (35 g/l water) until the movements of the gills ceased. The jaw was cut open from the ventral side and a cut was made through the epithelium of the palate. With a dental drill an opening was made in the palate just below the pituitary. Under a dissecting microscope the outer meninx was opened between the two branches of the *arteria encephalica*, rostrally or caudally to its anastomosis, in order to reach the brain. The lesions in the brain were performed with a specially designed scalpel and were placed at different levels of the hypothalamus (see results). The wound in the jaw was sutured with silk thread, while the opening in the palate was left untreated—it soon became covered with mucus.

After the operation the eels were placed on a white continuously illuminated background for 2–7 days. Sham-operated controls were not utilized, because lesions in a number of hypothalamic sites did not effect the color change mechanisms (Fremberg, unpublished).

### 3. Histochemical Procedure

After decapitation, the brain with the pituitary attached was rapidly dissected out and frozen in liquid nitrogen with propanebutane as intermediate agent. Freeze-drying (cold finger type) and subsequent formaldehyde vapor treatments were performed according to Falck and Owman (1965). The majority of the brains were treated at 80°C for one hour with paraformaldehyde equilibrated in air with a relative humidity of about 50%, followed by rapid embedding in paraffin in vacuo. A few brains were treated with a higher relative humidity and for a longer time. The histochemical procedure according to Håkanson and Sundler (1974) was employed on five brains and the procedure according to Lorén et al. (1976) on five other eel brains.

The brains were serially sectioned at 5–6 µm, mounted using liquid paraffin and examined with a Zeiss or Leitz fluorescence microscope provided with a BG 12 as the primary filter and a Zeiss 47 + 50 or K 530 as the secondary filter.

Control brains were treated as above, with the omission of the formaldehyde treatment. The specificity of paraformaldehyde fluorescence was tested as recommended by Björklund et al. (1972 b).

### 4. Microspectrofluorometric Technique

Emission and excitation measurements were recorded with a microspectrophotometer MS 1206, Beckman Instruments (München), modified for fluorescence assay. In emission measurements a Schott UG 1 (2 mm) acted as primary or excitation filter. The secondary or barrier filter Schott KV 418

was used in combination with a Schott BG 38 (4 mm) to eliminate the red component of the exciting light. In emission and excitation measurements normally performed with a spectral band width of 10 nm, the same recording (unchanged signal amplification and measuring diaphragm) was made on a "blank" tissue after each analysis of a specific fluorescent structure as near as possible to the analyzed element. The result of a point to point subtraction of these two spectra was corrected with factors for each wavelength selected. Further technical data concerning the instrument and the technical procedures have been dealt with previously (Hartwig, 1975; cf. Calas et al., 1974).

Finally, all spectra were given as corrected instrument readings expressed as relative quanta plotted against wavelength according to Björklund et al. (1972b). As recommended by Björklund et al. (1968, 1972a) a two step HCl vapor treatment was performed to differentiate the primary catecholamines, noradrenaline (NA) and dopamine (DA), in the following way: the non-deparaffinized sections mounted on quartz cover slips were exposed to the vapors of a fresh solution of concentrated HCl at room temperature for periods of 5 and 15 seconds in a closed Petri dish. The embedding medium (paraffin) was not removed as this helped to guard the specimen against mechanical destruction and the influences of air humidity.

## Results

In the teleosts, disagreement still exists concerning the exact border between the telencephalon and the diencephalon. The following delimitation is employed in the present study: ventrally, the border is placed between the *pars parvocellularis* of the preoptic nucleus and the postoptic commissural region, the latter being dominated by the *commissura transversa*. Dorsally, the border is at the *velum transversum*.

According to this definition the *pars parvocellularis* of the preoptic nucleus (NPOpp) belongs to the telencephalon and the *pars magnocellularis* to the diencephalon. It may be noted that the terminology used in the present paper for the ventricular system is adapted to that of Baumgarten and Braak (1967) in the goldfish.

It is worthwhile to mention that in the eel the optic nerves decussate outside the brain and rather far frontally; therefore, the true *chiasma opticum* is situated at the level of the *commissura anterior*. Thus, the optic tracts actually first enter the brain at the frontal-most part of the diencephalon (compare Fig. 1).

Specific fluorescence occurs in the following areas of the telencephalon and diencephalon (see Fig. 1):

### *Telencephalon*

A fluorescent "*nucleus telencephali posterior*", described by Lefranc et al. (1969, 1970), could not be verified in the present study. The *nucleus recessus praeoptici*, on the other hand, exhibits a strong monoamine fluorescence (Figs. 1 and 2). However, this fluorescence seems to be localized in terminals and/or in preterminals, although the presence of fluorescent perikarya cannot be definitely excluded. Lefranc and L'Hermite (1972) described tracts leaving the area of the *nucleus recessus praeoptici* partly in an antero-dorsal direction and partly in a latero-dorsal direction. Distally all fibers seem to disappear in the NPOpp region. These two monoamine tracts, probably running to the *nucleus recessus praeoptici* and not terminating in the NPO area, were confirmed in this investigation.

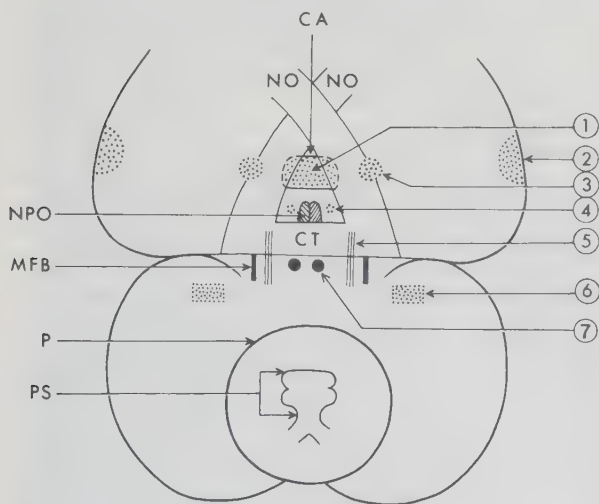


Fig. 1. Telencephalon and diencephalon of the eel, *Anguilla anguilla*. Ventral view. Dotted areas indicate catecholamine fluorescence of preterminals and terminals. For the monoamine perikarya of the paraventricular organ, see Figures 13 and 14. The following details are shown in a horizontal projection: CA commissura anterior; CT commissura transversa; MFB medial forebrain bundle; NO nervus opticus; NPO nucleus praepopticus; P pituitary; PS pituitary stalk; 1 nucleus recessus praepoptici; 2 catecholamine fluorescence in the lateral part of the telencephalon; 3 nucleus entopeduncularis; 4 catecholamine fluorescence lateral to the pars parvocellularis of the preoptic nucleus; 5 catecholamine fluorescence on the border between telencephalon and diencephalon; 6 nucleus lobi inferioris; 7 nucleus hypothalami anterior (catecholamine perikarya)

Three other telencephalic areas show a concentration of monoamine fluorescence (Fig. 1). One area is situated laterally in the hemispheres. However, its position is much more lateral than that of the *nucleus telencephali posterior* described by Lefranc et al. (1969). In most cases a second area can be observed in the region of the *nucleus entopeduncularis* (Kappers et al., 1960) exhibiting a weak fluorescence. The third area is situated laterally to the NPOpp (Figs. 5, 8).

### Diencephalon

*A. Monoamine-Containing Nuclei.* Fluorescent perikarya in the geniculate body can not be identified, and thus evidence for a fluorescent *nucleus diencephali posterior* (Lefranc et al., 1969) is not obtainable. However, approximately 12 catecholamine-containing cells, not yet described, can be found ventrally in the anterior part of the hypothalamus (Figs. 10, 11). They are located bilaterally just posterior to the commissura transversa. This nucleus will be termed the *nucleus hypothalami anterior* (NHA) in this study. Since this nucleus is found only in a limited number of specimens, a microfluorometric analysis of the amine content of these perikarya could not be performed.

The distribution of specific fluorescent structures contacting the cerebrospinal fluid of the diencephalic ventricle or located within a short distance from the ventricular border is generally in agreement with the description given by Lefranc et al. (1970) and L'Hermite and Lefranc (1972). In the present paper, however, the term paraventricular organ (PVO) will be used for the whole complex. A series of transverse sections is presented in order to explain its appearance in more detail (Figs. 13, 14). Very strong fluorescence is found around all parts of the *recessus posterior*, forming the *nucleus recessus posterioris* (NRP, Figs. 15f, 14f). The fluorescence in connection with the *recessus lateralis*, forming the *nucleus recessus lateralis* (NRL), is mostly situated around dorsal ependymal furrows (Figs. 13c, 14c); frontally these furrows take a more medial position. Further posteriorly, these furrows are absent and the fluorescent area is more expanded. The fluorescent cells in this region are found dorsally and ventrally to the recess (Figs. 13d, 14d). Within a short distance frontally, at the level of a prominent blood vessel, fluorescent cells are not observed in relation with the recess (Fig. 13b–c).

In the region where the third ventricle expands dorsally, fluorescent cells can be seen in two curvatures of the ventricular wall (Figs. 13a–d, 14b), and a little more frontally these fluorescent cell areas become continuous with the fluorescence of the *recessus lateralis* (Figs. 13a–b, 14a). This part was called *nucleus parvocellularis* of the “*gouttière dorsale du 3<sup>e</sup> ventricule*” by Lefranc et al. (1970); it will be called PVO *pars anterior* in the present paper. Almost directly in front of this area the fluorescent perikarya bordering the ventricle can no longer be recognized, so that the continuously fluorescent ventricular wall exhibits a sharp frontal border. Thus the PVO *pars anterior* is distinctly separated from the NRL by a small area devoid of fluorescent perikarya (Fig. 13b–c).

**Figs. 2–9.** Catecholamine fluorescence in the telencephalon and diencephalon of the eel. *V* third ventricle. Scale markers 50  $\mu$ m

**Fig. 2.** Nucleus recessus praepoptici. Sagittal section, rostral end to the left. Note the high fluorescence intensity of preterminals (*open triangles*) and the lower intensity of terminals (*arrows*). Fluorescent perikarya cannot be excluded with certainty. Fluorescence induced according to Lorén et al. (1976)

**Fig. 3.** Habenular complex. Horizontal section. Ventricle to the right. Note the area of densely innervated perikarya (*open arrows*) and the region devoid of nerve cell perikarya (*filled arrow*)

**Fig. 4.** Habenular complex. Frontal section. In comparison to Figure 3 opposite side

**Fig. 5.** Pars parvocellularis of the preoptic nucleus (*NPOpp*). Frontal section. Note the high fluorescence intensity lateral to the *NPOpp*

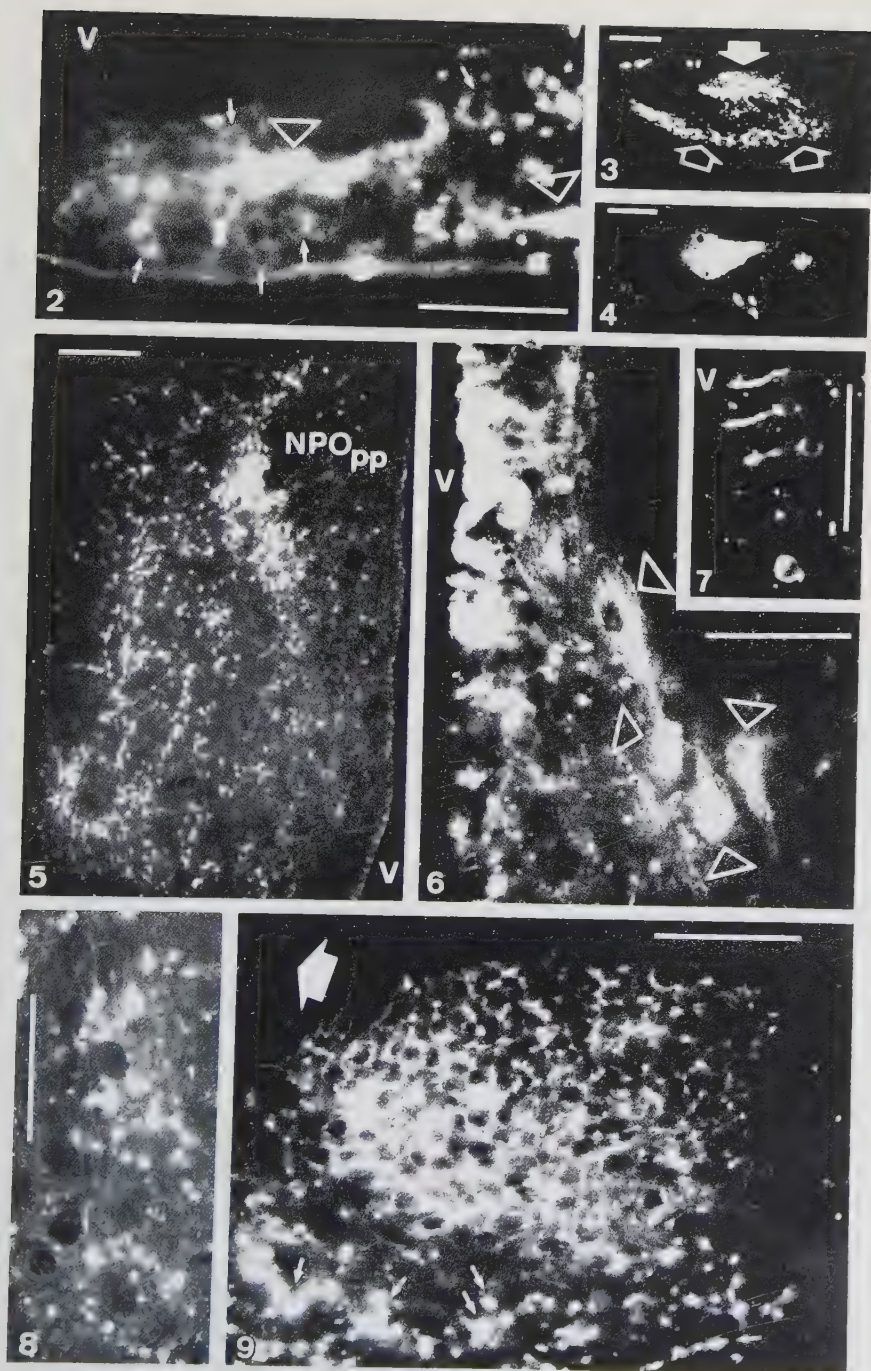
**Fig. 6.** Pars anterior of the paraventricular organ (*PVO*) and PVO-accompanying cells (*open triangles*). Frontal section. Fluorescence induced according to Håkanson and Sundler (1975)

**Fig. 7.** Single PVO cells showing dendritic processes directed to the ventricular lumen. Frontal section

**Fig. 8.** Catecholamine innervation of the preoptic nucleus. Frontal section

**Fig. 9.** Nucleus lobi inferioris. Frontal section. Note the high density of terminals and the higher fluorescence intensity of preterminals neighboring the NIL (*arrows*). The *large arrow* points to the recessus lobi inferioris of the third ventricle





Lateral to the *nucleus parvocellularis* of the "gouttière dorsale du 3<sup>e</sup> ventricule" of Lefranc et al. (1970), large green-fluorescent cells appear which apparently have no direct contact with the ventricle (Fig. 6). These cells have been called "*nucleus magnocellularis*" by Lefranc et al. (1970) and "big fluorescent cells" by Ekengren (1975), and PVO-accompanying cells by Terlou and Ploemacher (1973). Contrary to the figure shown by Lefranc et al. (1970), these cells appear to occupy a considerable area. Finally, it should be mentioned that in the area between the *recessus posterior* and the *recessus lateralis* fluorescent cells can be seen bordering the third ventricle, which is very narrow at this point. This part will be called PVO *pars isthmi* (Figs. 13e, 14e).

In addition to these fluorescent perikarya a strong concentration of monoamine fluorophores is seen located in two areas not described before. One area is situated immediately rostral to the *recessus lobi inferioris* (Fig. 1). This area, characterized by a sharp border, possesses an oval shape both in the transversal and in the sagittal planes and contains several layers of small perikarya. However, the observed monoamine fluorescence seems to depend on fine, densely packed terminals (Fig. 9) although the presence of fluorescent perikarya cannot be excluded. This fluorescence appears green rather than yellow. It is more diffuse than the catecholamine fluorescence in the preterminals of the neighboring area (see Fig. 9). The latter will be termed *nucleus lobi inferioris*.

The second fluorescent area can be observed in the habenular region. This area exhibits an intense green-yellow fluorescence. The shape of the fluorescent area has a rather striking appearance (Figs. 3 and 4). It occupies the antero-ventral part of the habenulae; thus, it is situated ventral to the habenular commissure. The fluorescent area can be divided into an anterior and a posterior part. The anterior part is situated in a region rich in perikarya; the fluorescence forms a crescent

**Figs. 10–13.** Catecholamine fluorescence in the diencephalon of the eel. Frontal sections. *V* third ventricle. Scale marker Figure 11 – 10  $\mu\text{m}$ ; other scale markers 100  $\mu\text{m}$

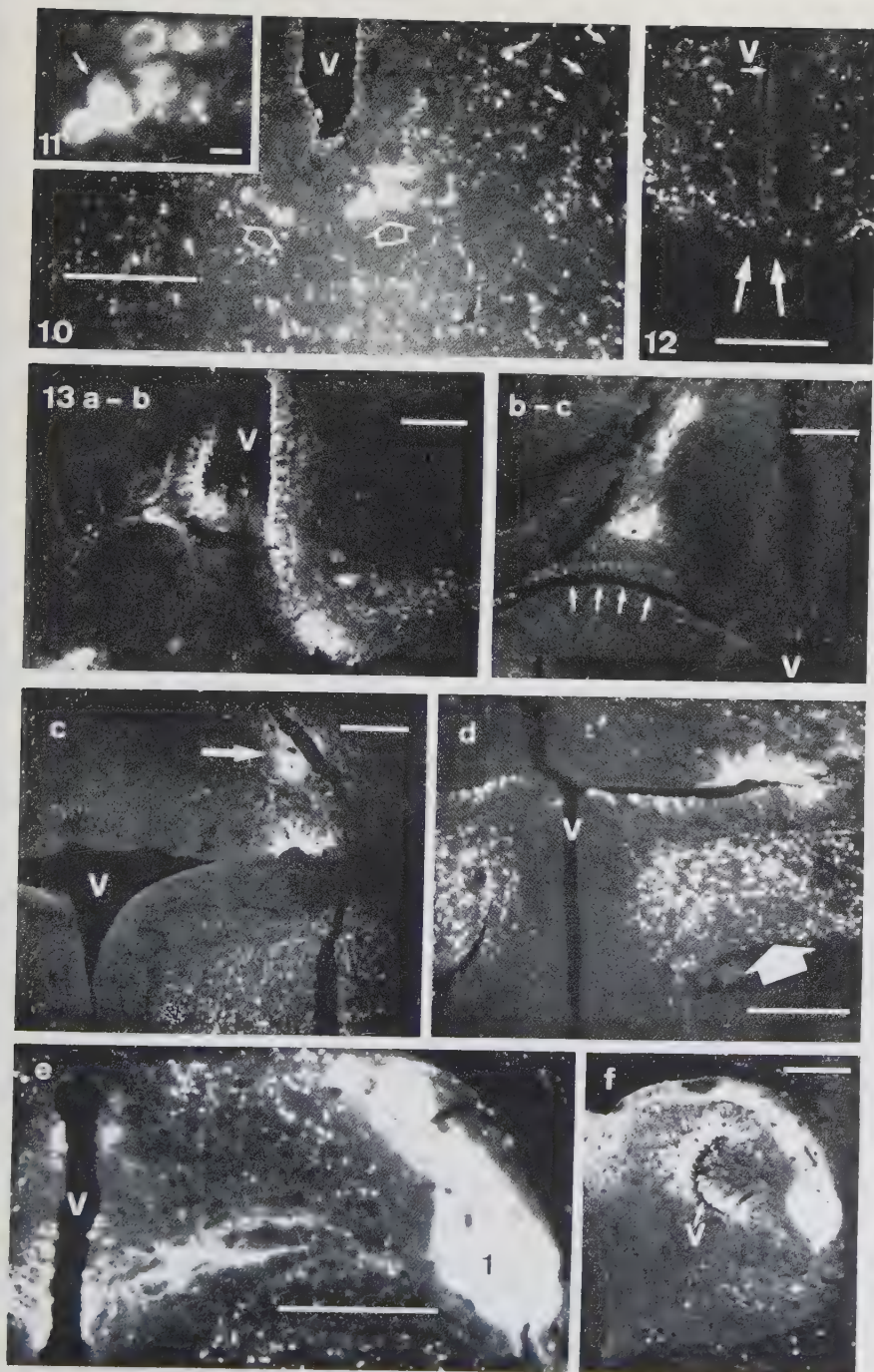
**Fig. 10.** Nucleus hypothalami anterior (*open arrows*). Frontal section 100  $\mu\text{m}$  caudal to the center of the commissura transversa. *Small arrows* indicate commissural fibers

**Fig. 11.** Perikarya of the nucleus hypothalami anterior. Note the perikaryon probably encompassed by fluorescent terminals (*arrow*)

**Fig. 12.** Basal hypothalamus approximately 300  $\mu\text{m}$  caudal to the commissura transversa. Note the absence of fluorescent structures in the region occupied by neurosecretory tracts (*arrows*)

**Fig. 13a–f.** Series of frontal sections showing the distribution of amine fluorescence bordering the third ventricle (compare with Fig. 14). Lines a–f in Figure 15 indicate approximate levels of cross sections shown in Figure 13a–f. **a–b** Pars anterior of the paraventricular organ (PVO), oblique section, with the effect that the right side corresponds to Figure 14a while the left side corresponds to Figure 14b. **b–c** Fiber tracts dorsolateral to the PVO. Note the absence of fluorescent PVO perikarya at this level. *Arrows* indicate rostral part of the recessus lateralis of the third ventricle. **c** Rostral part of the nucleus recessus lateralis, occupying most of the area between line b and d in Figure 14. *Arrow* indicates fiber tract dorsal to the nucleus recessus lateralis (compare Fig. 13b–c). **d** Posterior part of the nucleus recessus lateralis. Note the presence of fluorescent perikarya dorsal and ventral to the recessus lateralis. **e** Pars isthmi of the PVO. *l* indicates fiber tract dorsolateral to the PVO. **f** Nucleus recessus posterioris





which follows the anterior wall of the habenulae. It cannot be definitely excluded that part of this fluorescence is due to perikarya. The posterior part is, on the other hand, situated in an area devoid of perikarya. A connection can be seen between these two fluorescent regions (Fig. 3). The source and/or the destination of the fluorescent fibers are open to discussion. A few fibers can, however, be seen approaching and/or leaving the posterior part in a straight posterior direction as well as in a ventral direction.

Finally, specific fluorescent cells are observed in the epiphysis cerebri. Findings dealing with the epiphysis cerebri of the eel will be published elsewhere.

**B. Monoaminergic Tracts and Pituitary Innervation.** A description of the monoaminergic tracts in the diencephalon of the eel was given by L'Hermite and Lefranc (1972). They described tracts from the NRP innervating other parts of the PVO as well as the NLT, tracts from the PVO pars anterior innervating the NOPpp. Other distinct tracts connect the brain stem with the *lobi inferiores*. Some findings can be added to this description. A very strong concentration of fluorescent fibers is found in the area around the border between the telencephalon and diencephalon (Fig. 1). These fibers are situated bilaterally and have a rather ventral position.

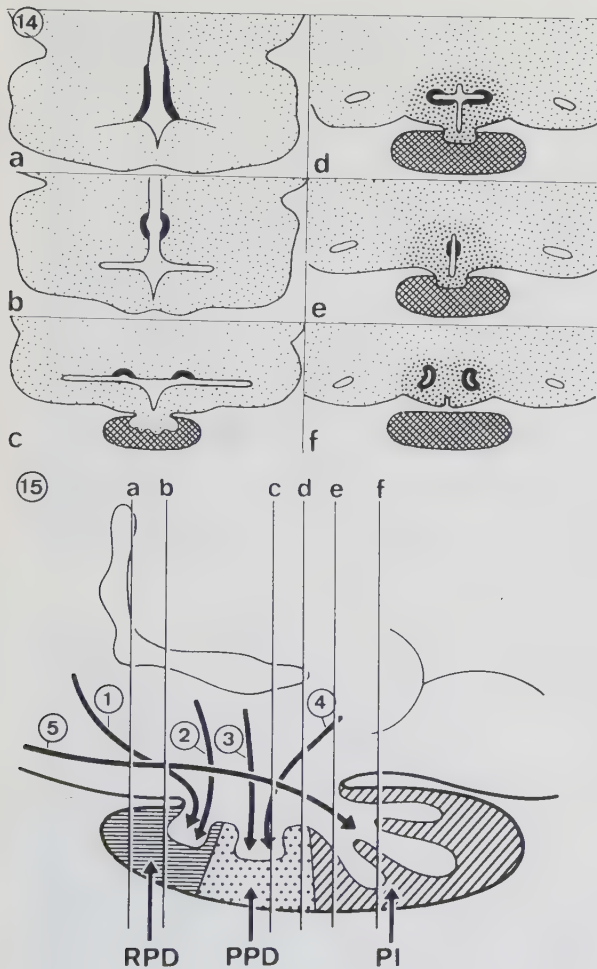
In an attempt to clarify the innervation of the pituitary, mainly that of the NIL, some lesion experiments were performed. Lesions in the hypothalamus of the eel (Fremberg, unpublished) show that a tentative inhibiting pathway (not identical with the classical neurosecretory tract) to the NIL enters the pituitary frontally; this pathway runs a rather superficial course between the commissura transversa region and the pituitary stalk. Therefore, lesions were placed at different levels in the hypothalamus in order to induce an accumulation of monoamines in this pathway. The innervation pattern of the pituitary, as could be analyzed from intact and lesioned material, is as follows (see Figs. 15–19):

**Rostral Pars distalis (RPD).** Within the RPD the monoaminergic fibers of the neural lobe seem to enter this lobe partly from a rostral direction and partly from a dorsal direction (Fig. 15, tracts 1 and 2). It was impossible to trace with certainty these tracts back to their origin, but it seems that the nerves entering from rostral direction originate in the PVO, *pars anterior*. On the other hand, the fibers entering dorsally seem to originate from the fluorescent area around the *recessus lateralis*. Immediately before entering the stalk, these fibers seem to take a more lateral position.

**Proximal Pars distalis (PPD).** Within the PPD the neural lobe appears to receive its monoamine supply from dorsal and posterior directions (Fig. 18; tracts 3 and 4 in Fig. 15). Again, the origin of these fibers can not be located with certainty, however, some findings indicate that the dorsal tract originates in the *nucleus recessus lateralis*, while the posterior component seems to originate in the fluorescent area around the *recessus posterior*. The last-mentioned tract takes a more medial position in the stalk than the dorsal tracts.

**Neurointermediate Lobe (NIL).** In the medial-most region of the stalk few fluorescent fibers occur. A distinct rather narrow tract, however, can be followed from a point in the hypothalamus, approximately at the level of the RPD, into the stalk





**Fig. 14a-f.** Series of schematic cross sections showing the distribution of monoamine fluorescence bordering the third ventricle and its recessus (*black areas*) (Compare with Fig. 13). Lines labeled a-f in Figure 15 mark the level of the presented cross sections. **a** Pars anterior of the PVO. Dorsal extension of the third ventricle, frontal entrance to the recessus lateralis. **b** Pars anterior of the PVO. Indentation of the ventricular border. **c** Anterior part of the *nucleus recessus lateralis*. **d** Posterior part of the *nucleus recessus lateralis*. **e** Pars isthmi of the PVO. **f** *Nucleus recessus posterioris*

**Fig. 15a-f.** Diagrammatic sagittal section showing the proposed monoamine innervation pattern (1-5) of the different parts of the pituitary (for details, see p. 10). Lines labeled a-f indicate level of cross sections presented in Figures 13 and 14. **RPD** rostral pars distalis of pituitary; **PPD** proximal pars distalis of pituitary; **PI** pars intermedia of pituitary encompassing neural lobe processes

area. Anteriorly, the tract runs closely to the brain surface, but upon entering the stalk area it runs slightly deeper, and then makes a ventral bend into the stalk (Figs. 16, 19; tract 5 in Fig. 15). Its further course seems to be mainly directed towards the NIL, because after lesions between the *pars distalis* and the NIL an accumulation of transmitter substance can be observed (Fig. 17), and the tract can also be followed more caudally than normal. In transverse sections fluorescent fibers can be seen close to (lateral and dorsal) the NS-tract in the posterior hypothalamus (Fig. 12). The origin of this tract is difficult to establish, but it appears that it takes a more lateral position and becomes bilateral in the anterior hypothalamus. The tract can then be observed rather far rostrally, especially after lesions. Sometimes after lesions in the anterior hypothalamus, an accumulation of amines can be observed in the region of the *commissura transversa* both rostral and distal to the lesion.

After hypothalamic lesions the fluorescence in the NIL is generally very strongly reduced, or, as in some animals, disappears completely, in combination with a darkening of the animal. This phenomenon is also observed after lesions performed near the *commissura transversa*. The fluorescence within the *pars distalis* is, on the contrary, seldomly affected by lesions in the antero-ventral hypothalamus.

### *Spectral Analysis of Fluorescent Compounds*

The fluorescent compounds found in the diencephalon of 15 eels showed excitation and emission maxima of primary catecholamine fluorophores (emission maximum at 480 nm; a high excitation peak at 410 nm, accompanied by a lower one at 320 nm; Fig. 20) and of 5-hydroxytryptamine 5-hydroxytryptophane (5HT 5HTP) fluorophores (emission maxima between 520 and 530 nm; excitation maximum at 390 to 400 nm accompanied by a lower peak at 320 nm; Fig. 21). 5HT 5HTP fluorophores could be identified only in approximately 20% of the PVO nerve cells. The highest fluorescence intensity of these fluorophores was located in the dendritic intraventricular protrusions of PVO neurons. 5HT 5HTP containing fibers, preterminals or terminals were not found in any of the analyzed brain areas. In contrast to the catecholamine fluorophores, the indolamine fluoro-

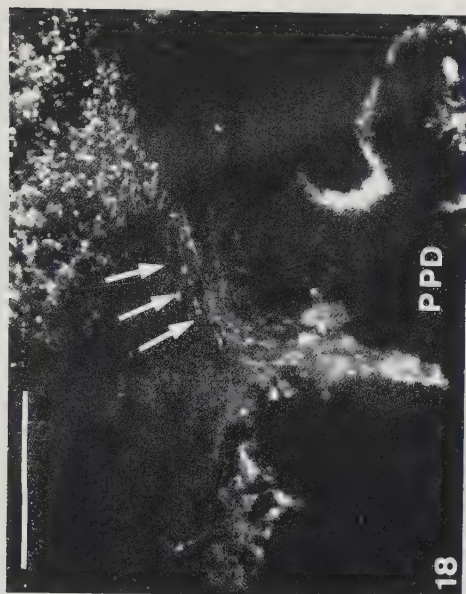
**Figs. 16–19.** Sagittal sections showing the catecholamine innervation of the pituitary (compare with Fig. 15). *RPD* rostral pars distalis of pituitary; *PPD* proximal pars distalis of pituitary; *NIL* neurointermediate lobe; *V* third ventricle. *I* nucleus recessus posterioris; *2* pars anterior of the paraventricular organ. Scale markers 100  $\mu$ m

**Fig. 16.** Parasagittal section showing the medial monoamine tract from the rostral-most part of the pituitary (*large arrow*) to the proximal end of the pituitary stalk (*small arrow*)

**Fig. 17.** Parasagittal section at a more lateral level than in Figure 16. Animal lesioned between pars distalis and neurointermediate lobe; pituitary dislocated somewhat caudally. Accumulation of fluorescent material indicates a monoamine-containing tract entering the NIL from a rostral direction (*arrows*)

**Fig. 18.** Monoamine-containing fibers entering the neural lobe (intercalated with the PPD) from a caudal direction (*arrows*)

**Fig. 19.** Monoamine-containing fibers entering the rostral part of the pituitary stalk in S-shaped line (*marked by dots*). A part of these fibers probably run to the NIL



phores exhibited a high fading rate (more than 50% decrease of fluorescence intensity within 5 min) due to irradiation with light at the most effective wavelength (390 nm).

The results of microfluorometric investigations in a two step procedure with HCl vapor-treated brain sections (fluorometric differentiation between NA and DA fluorophores) are summarized in Table 1. In HCl vapor-treated NA fluorophores characterized by a 370:320 nm excitation peak ratio of 0.3–0.85, a 5 min irradiation with the most effective wavelength (380 nm) resulted in a reduction of 70% of fluorescence intensity, whereas in DA fluorophores with a 370:320 nm excitation peak ratio of 1.1–1.6 the fluorescence intensity was reduced only by 45% (see Fig. 22). In fluorescent perikarya, preterminals and terminals with a 370:320 nm excitation peak ratio between 0.9 and 1.05 irradiation with the most effective

**Table 1.** Number and results of spectral recordings in order to differentiate noradrenaline from dopamine in various brain areas (two step HCl vapor treatment). Microfluorometric results in monoamine-containing protein microdroplets (Hartwig and Lyncker, unpublished; compare Björklund et al., 1972a) indicate that an excitation peak ratio 370:320 nm  $\leq 0.85$  is characteristic for noradrenaline fluorophores, whereas values of 370:320  $\geq 1.1$  are typical for dopamine fluorophores. 370:320 nm peak ratios between 0.86–1.09 observed in model studies speak in favor for NA/DA fluorophore mixtures. In tissue studies these values might be due to a poor signal to noise ratio in measurements of delicate fluorescent terminals

|                                                                    | 370:320 $\leq 0.85$ (NA)                        | 370:320 = 0.86–1.05 | 370:320 $\geq 1.1$ (DA) |
|--------------------------------------------------------------------|-------------------------------------------------|---------------------|-------------------------|
| NPOpp area<br>(preterminals and terminals)                         | 27                                              | 4                   | 6                       |
| PVO (perikarya and<br>intraventricular protrusions)                | —                                               | —                   | 15                      |
| PVO (dorsolateral fiber tract)                                     | —                                               | —                   | 7                       |
| PVO (preterminals and terminals)                                   | 8                                               | —                   | 7                       |
| PVO accompanying cells                                             | 12                                              | 1                   | 4                       |
| <i>Nucleus recessus praeoptici</i><br>(preterminals and terminals) | 21                                              | 12                  | 17                      |
| <i>Nucleus lobi inferioris</i><br>(preterminals and terminals)     | 9                                               | 1                   | 21                      |
| <i>Lobus inferior</i><br>(preterminals and terminals)              | 40                                              | 4                   | 3                       |
| Neurointermediate lobe<br>(terminals)                              | 19                                              | 30                  | 22                      |
| PPD and RPD (terminals)                                            | results comparable to NIL, only 10 measurements |                     |                         |
| Habenular complex (terminals)                                      | 27                                              | 12                  | 17                      |



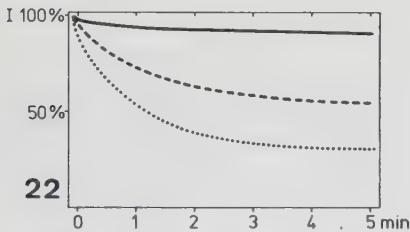
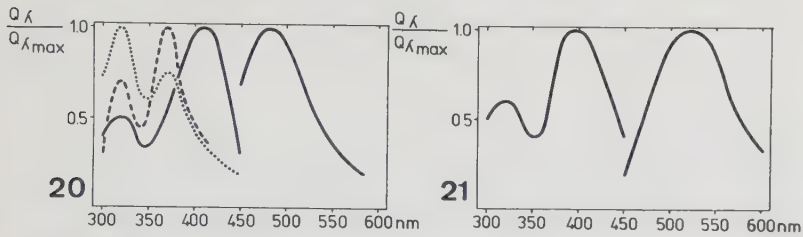


Fig. 20. Fluorescence emission (right) and excitation (left) spectra of catecholamine fluorophores (compare with Table I): — after formaldehyde treatment only; ---- excitation spectrum of dopamine fluorophores after an additional stepwise treatment with HCl vapor; ..... excitation spectrum of noradrenaline fluorophores after an additional stepwise treatment with HCl vapor

Fig. 21. Fluorescence emission (right) and excitation (left) spectra of yellow-fluorescent perikarya of the paraventricular organ

Fig. 22. Photodecomposition of catecholamine fluorophores. Measurements performed with the most effective wavelengths: — after formaldehyde treatment only; ---- photodecomposition of dopamine fluorophores after an additional HCl vapor treatment; ..... photodecomposition of noradrenaline fluorophores after an additional HCl vapor treatment

wavelength induced within 5 min a fluorescence intensity reduction of approximately 55%. Thus, three different types of fluorophores are observed after HCl vapor treatment: type 1 showing spectral data of NA fluorophores, type 2 exhibiting spectral characteristics of DA fluorophores; and type 3 showing uncharacteristic spectral data.

## Discussion

### *Telencephalon*

A monoamine-containing nucleus in connection with the preoptic recess has been observed in *Anura* (preoptic recess organ; Terlou and Ploemacher, 1973; Prasad Rao and Hartwig, 1974), and in *Scyliorhinus canicula* (*organum vasculosum praeopticum*; Wilson and Dodd, 1973). However, in contrast to the *nucleus recessus*

*praeoptici* of *Leuciscus rutilus* (Ekengren, 1975), this nucleus in *Anura* and *Scyliorhinus* consists of cerebrospinal fluid-contacting neurons located mainly in the lateral wall of the preoptic recess; in *Leuciscus* the nucleus is situated in the floor of the preoptic recess, but is not cerebrospinal fluid-contacting. In *Rana temporaria* (Prasada Rao and Hartwig, 1974) these neurons contain DA, while in the eel the terminals and preterminals in the floor of the preoptic recess are characterized by DA and NA fluorophores (Table 1).

In the telencephalon mainly NA fibers are found, probably originating in NA perikarya of the diencephalon ("PVO accompanying cells"), or of the lower brainstem. Lefranc et al. (1970) described in the eel a *nucleus myelencephali dorsolateralis*. This nucleus has been confirmed in the present study to contain NA perikarya, probably corresponding to the *locus coeruleus* in mammals and birds (see Björklund et al., 1974).

NA afferents to the telencephalon and the Gomori-positive neurosecretory cells arising from the lower brainstem have been suggested in *Xenopus laevis* (Terlou and Ploemacher, 1973) and in mammals (for review, see Lindvall and Björklund, 1974). Additionally, there seems to be a contribution of DA fibers to the telencephalon of the eel, especially to the *nucleus recessus praeoptici* and to the area lateral to the NPOpp. A source of these DA fibers might be the PVO (compare findings in *Xenopus*; Terlou and Ploemacher, 1973).

### Diencephalon

*Circumventricular Fluorescence.* The fluorescent material seen around the diencephalic ventricle seems to have a similar distribution as in other lower vertebrates, but its morphological appearance varies with the shape of the ventricular system (Fig. 14). Discrepancies in the nomenclature of these fluorescent areas exist both within and between different classes of vertebrates. According to Vigh (1971) the most appropriate term for the whole fluorescent complex in the eel is paraventricular organ (PVO) which then can be divided into subunits. As the terms *nucleus recessus lateralis* (NRL) and *nucleus recessus posterioris* (NRP) are strongly established for the teleosts investigated so far with the Falck-Hillarp method, it appears expedient to retain these terms for the fluorescent areas bordering the *recessus lateralis* and the *recessus posterior* of the third ventricle. For the other two parts not described in other teleosts, the terms *pars anterior* and *pars isthmi* of the paraventricular organ are proposed (Fig. 14).

The PVO in the eel is similar to the *organum vasculosum hypothalami* of *Scyliorhinus* (Wilson and Dodd, 1973) and to both the PVO and NID in *Anura* (Terlou and Ploemacher, 1973; Prasada Rao and Hartwig, 1974). It is worthwhile to mention that in the eel the DA and 5HT/5HTP perikarya of the PVO receive a high input of NA afferents. NA fluorophores are found in the area of axonal processes of the PVO perikarya. Similar findings have been reported by Nozaki et al. (1975) in the Japanese quail.

*Lobi inferiores.* It seems most probable that the oval shaped concentration of fluorescence in the *nucleus lobi inferioris* is due to densely packed terminals, although the existence of fluorescent perikarya sometimes could not be excluded.

Densely packed amine terminals might give the impression of fluorescing perikarya, especially if the fluorophores tend to diffuse. A nuclear area, *nucleus lobi inferioris*, exhibiting monoamine fluorescence exists in *Scyliorhinus* (Wilson and Dodd, 1973).

*Habenulae.* Monoamine fluorescence in the habenular region has been found in *Rana temporaria* (Zimmermann and Paul, 1972) and in the rat (Wiklund, 1974). In the rat the monoamine fluorescence is due to 5HT perikarya and NA fibers, whereas in the eel only DA and NA fluorophores could be identified; 5HT could not be demonstrated even after pharmacological treatment of eels according to Aghajanian et al. (1973; compare Wiklund, 1974).

Whatever the true nature of the fluorescence in the habenulae of the eel may be, the existence of specific monoamine fluorescence in this particular brain area is of great interest in view of the manifold interrelation between the habenulae and other brain areas (Rausch and Long, 1971).

*Pituitary Innervation.* The innervation of the pituitary is poorly investigated in teleosts. Baumgarten and Braak (1967) mentioned that a fiber tract from the NRP runs to the pituitary stalk in the goldfish, and Ekengren (1975) mentions a tract entering the pituitary stalk from a frontal direction. The pituitary of the eel is innervated from different sources (see Fig. 15), but it was impossible to establish the origin of these different fibers with certainty. However, regarding the direction from which the different parts of the pituitary are innervated and the results of the microspectrofluorometric investigation, some speculation on the origin may be postulated.

The neural lobe processes within the *pars distalis* seem to contain both DA and NA. The DA fibers in the *pars distalis* appear to originate mainly in the PVO. Both parts of the *pars distalis* seem to receive nerves from the NRL, and this assumption is supported by an investigation of Urano (1971) on the distribution of monoamine oxidase (MAO) in the eel brain. Urano reported that two fiber tracts run in a straight dorso-ventral direction from the *nucleus lateralis tuberis* (NLT) to the two parts of the *pars distalis*. As there is no evidence for the existence of monoamine perikarya in the NLT, these tracts probably have their origin in the NRL. The tracts from the NRL curve laterally before proceeding in a ventral direction, which may give the impression that they originate in the NLT. Furthermore, it is also possible that a portion of the fibers from the PVO have their terminals in the NLT area (Ekengren, 1975), i.e. masking the perikarya.

It can be seen that the PPD is partly innervated from a posterior direction, and the course of these fibers evidently indicates that they originate in the NRP, whereas the fibers innervating the RPD from a rostral direction arise in the *pars anterior* of the paraventricular organ. A crescent of fibers from this region converging into the pituitary stalk is evident in sagittal sections.

The NA nerves in the *pars distalis* most probably have their origin in the lower brainstem (myelencephalon), or in the autonomous nervous system. However, noradrenaline perikarya have been identified in this investigation not only in the lower brainstem, but also the PVO accompanying cells most probably contain NA. As the lateral basal hypothalamus predominantly contains NA, it appears that NA fibers enter the *pars distalis* more laterally than the DA fibers.

The precise source of the innervation of the NIL, which was the main objective of this investigation, remains to be elucidated. The microspectrofluorometric investigation has shown that in the NIL of 100 analyzed preterminals and terminals, 27 exhibited the spectral characteristics of NA, 31 had a 370:320 nm excitation peak ratio of DA, and 42 could not be identified. The real content of DA, which might be higher or lower than that of NA, can be measured only using biochemical procedures.

The main function of the *pars intermedia* of the eel is to produce melanophore-stimulating-hormone (MSH), and the release of this hormone seems to be under inhibitory control of CA nerves (Fremberg and Olivereau, 1973; Fremberg and Meurling, 1975; Fremberg, unpublished results). On the basis of the microspectrofluorometric investigation, it is impossible to suggest whether this control of MSH release is mediated by DA or NA, as their amounts are similar. General data concerning the cellular regulation of MSH release have been dealt with by Hadley et al. (1975). In most mammals investigated the PI predominantly contains DA, but also NA, and it has been seen in the rat that the PI cells are surrounded by DA terminals (Björklund, 1968). In Anura the precise role of the CA nerves innervating the PI cells is not known; however, in the PI of Anura, both DA and NA fluorophores have been detected (Prasada Rao and Hartwig, 1974).

The lesion experiments and the subsequent fluorescent-microscopical investigations have made it clear that the NIL, at least for the most part, is innervated from a rostral direction by a medial tract, which could be followed to the commissura transversa region, taking a more lateral course probably becoming bilateral further rostrally. This is in accordance with the investigation by Urano (1971), in which a medial MAO positive tract could be followed from the commissura transversa region into the NIL. It seems reasonable to assume that this medial aminergic tract is involved in the MSH release: (1) Lesion experiments (Fremberg, unpublished) show that a pathway involved in the inhibition of MSH release closely follows the course of the classical neurosecretory tract(s) in the hypothalamus, in accordance with the course of the medial fluorescent tract, and (2) an accumulation of fluorescent material is achieved in this medial tract after a lesion between the NIL and the *pars distalis*, accompanied by a darkening of the animals. It seems also reasonable to assume, based on the lesion experiments (Fremberg, unpublished) and from a comparative view (see below), that the MSH release inhibiting nerves originate in the telencephalon or diencephalon. So far only three aminergic nuclei, the NHA, the PVO and the PVO accompanying cells, have been found rostral to the lower brainstem, and thus there seem to be only three alternatives with respect to the origin of fibers inhibiting MSH release. From the lesion experiments, the NHA is a tempting alternative, but this will be more difficult to establish. As in *Xenopus* (Terlou and Ploemacher, 1973) the tracts from the PVO run rostral before proceeding in a posterior direction, and thus in lesion experiments tracts from the NHA and the PVO will be influenced by the same surgical procedure. In addition, the PVO and the PVO accompanying cells are very difficult to reach surgically without interfering with other tracts.

In other vertebrates different sources involved in the inhibition of MSH release have been suggested. In *Scyliorhinus* the aminergic innervation of the PI, involved in the inhibition of MSH release, has been traced back to a *nucleus medius*



*hypothalami*, situated immediately behind the *chiasma opticum*, thus on the same latitude in the hypothalamus as the NHA in the eel (Wilson and Dodd, 1973). The innervation of the PI of *Xenopus* is, on the other hand, said to originate in the dorsal diencephalon, in the PVO and/or the NID (Goos, 1969). Prasada Rao and Hartwig (1974) suggested that in *Rana temporaria* a tract from the preoptic recess organ innervates the PI, possibly together with a tract from the PVO. In the rat, it has been assumed that the tuberal DA-neurons of the arcuate nucleus innervate the PI cells (Björklund, 1968).

The part of the aminergic innervation of the intermediate lobe that is not involved in the inhibition of the MSH release may originate in the lower brainstem or in the autonomous nervous system, as well as in the PVO, the NHA or the PVO accompanying cells. There are several possible functions of these nerves; for example, innervation of blood vessels, regulation of other cell types in the PI (PAS+ cells), the release of neurosecretory substances or regulation of MSH synthesis. In mammals (Björklund, 1968) sympathetic NA nerves accompany blood vessels of the PI and the neural lobe.

### *Identification of Amines*

The observed excitation and emission recordings of formaldehyde-induced fluorophores are within the range found in monoamine-containing microdroplets (Hartwig and Lyncker, unpublished; see Björklund et al., 1972a). However, fluorophores of DOPA exhibit the same spectral characteristics as fluorophores of DA, and formaldehyde-induced fluorophores of adrenaline cannot be differentiated from NA fluorophores. Since DOPA, a precursor of catecholamine synthesis, is not to be expected in high concentrations in normal brain tissue (cf. Björklund et al., 1975) and considerable amounts of adrenaline are formed only after prolonged formaldehyde vapor treatment (Falck et al., 1962), the reported catecholamine spectra are most probably due to DA and NA fluorophores. Moreover, the findings of the present investigation are in good agreement with results of earlier microfluorometric and biochemical investigations on the hypothalamus of other lower vertebrate species (see Baumgarten, 1972; Juorio, 1973; Prasada Rao and Hartwig, 1974; Terlou and van Kooten, 1974).

In perikarya and terminals with a low fluorescence intensity a differentiation between NA and DA fluorophores is difficult due to a required high signal amplification and a poor signal to noise ratio. Recordings with a high signal amplification and mixed spectral data of NA and DA fluorophores have been classified as noncharacteristic results (Table 1). Moreover, in animals pretreated with monoamine oxidase inhibitors, mixed spectral recordings might be expected due to unlimited re-uptake of released monoamines.

In some specimens it could not be definitely decided whether the observed catecholamine fluorescence in the *preoptic recess* and in the *nucleus lobi inferioris* was due to perikarya or to densely packed terminals characterized by a diffusion of their fluorophore content. In order to overcome this problem, an application of ultrahistochemical procedures is required (cf. Richards and Tranzer, 1974).

Fish are characterized by the longest evolution period of all classes of vertebrates. Consequently, fish have developed a large scale of morphological and functional specializations (cf. Jollie, 1962). Due to a development among different lines, findings in brain anatomy reported in different teleost species are difficult to compare. However, the results of the present investigation are in agreement with the assumption that amine-containing perikarya and fiber tracts show a similar general morphological pattern and topographical arrangement in all classes of vertebrates: 1) Monoamine-containing perikarya located in the lower brainstem project to different telencephalic and diencephalic areas (ascending monoamine system; for review, see Lindvall and Björklund, 1974). Thus, complete hypothalamic deafferentation results in a marked loss of fluorescence intensity inside the hypothalamic island (*Rana temporaria*; Dierckx, 1974; *Coturnix coturnix japonica*; Nozaki et al., 1975; mammals: for review see Lindvall and Björklund, 1974). In the present study the existence of NA- and 5HT 5HTP-containing perikarya has been confirmed in the lower brainstem of the eel. 2) Monoamine-containing perikarya located in the hypothalamus predominantly exhibit short distance projections not leaving the diencephalon. The arcuate nucleus of mammals displays a cluster-like arrangement of peptidergic and dopamine-containing nerve cells. Further, in submammalian vertebrates the tuberal DA perikarya are lacking. However, submammalian vertebrates possess DA nerve cells in the paraventricular organ (for review, see Oksche, 1976). The latter is apparently missing in mammals.

Finally, lesions placed in the basal hypothalamus of the eel rostral to the pituitary stalk result in a darkening of the animal (increased MSH release). A MSH-release-inhibiting pathway seems to be a general property of the vertebrates (for review, see Prasada Rao and Hartwig, 1974; Terlou and Ploemacher, 1973). It penetrates into the pituitary stalk from its rostral aspect. However, the exact origin of this tract remains to be elucidated in future investigations.

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